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CHARACTERIZATION OF THE MOLECULAR AND IMMUNOREGULATORY PROPERTIES OF IGD-RECEPTORS EXPRESSED ON T LYMPHOCYTES

by

YAN WU

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

2000

This manuscript has been read and accepted for the Graduate Faculty in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

CHARACTERIZATION OF THE MOLECULAR AND IMMUNOREGULATORY PROPERTIES OF IGD-RECEPTORS EXPRESSED ON T LYMPHOCYTES

by

Yan Wu

Advisor: Professor Richard F. Coico

Since its discovery, the biologic role of immunoglobulin D (IgD) has remained enigmatic despite numerous experimental attempts to define its function. IgD is a predominant surface immunoglobulin (Ig) expressed in high density on mature, resting B lymphocytes along with IgM. Previous studies have shown that 1) receptors specific for IgD (IgD-R) are expressed by human $CD4^+$ and $CD8^+$ T cells whereas IgD-R appear to be restricted to the $CD4^+$ T cell population in mouse; 2) injection of oligomeric IgD in mice results in rapid increase in the number of $CD4^+$ $IgD\text{-}R^+$ T cells (~30%); 3) several other stimuli that activate T cells also induced upregulation of IgD-R; 4) in vitro studies have confirmed that cognate interactions between T and B cells are required to demonstrate enhanced helper activity using T cells with upregulated IgD-receptors (IgD-R). The goal of the current study was to assess the role of antigen-presenting B cells on $IgD\text{-}R^+$ T cell activation. We compared B cells from $IgD^{+/+}$, $IgD^{+/-}$, and $IgD^{-/-}$ mice to

determined whether IgD expression is prequisite for functional activity of IgD-R^+ T cells. Our results revealed that ligation of IgD on B cells and IgD-R on T cells promotes: 1) enhanced antibody responses and clonal expansion of antigen-specific T cells; 2) enhanced cytokine productions both at the protein and mRNA levels, with shift towards the T_H2 phenotype; 3) phosphorylation of intracellular proteins; and, 4) enhanced expression of CD28 on T cells. Molecular characterization of IgD-R demonstrated that a ~ 29 kDa band is phosphorylated and exhibits stong affinity for biotinylated IgD. In summary, our studies lead us to conclude that IgD-R are, indeed, involved in bi-directional signaling of B and T cells, and the skewing towards the T_H2 phenotype following crosslinking of the IgD-R is consistent with the functional properties of IgD-R^+ T cells which are known to significantly enhance antibody responses. Finally, studies of Fas antigen regulation and expression associated with upregulation of IgD-R suggest that ligation of IgD-R may protect T cells from undergoing apoptosis following their activation by IgD-expressing B cells. Therefore, IgD-R function not only as a ligand for IgD, but also play an important role in the regulation of humoral immune responses and T cell activation.

To

My Dear father, mother, sisters and my husband

For their loving support and inspiration.

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ABBREVIATIONS USED:

IgD	immunoglobulin D
IgD-R	immunoglobulin D receptor
mIgD	membrane IgD
IgM	immunoglobulin M
IgG	immunoglobulin G
Tδ	helper T cells bearing receptors for IgD
APC	antigen-presenting cells
GAM	goat anti-mouse
$IgD^{-/-}$	IgD-deficient mice
TD	thymus-dependent
DMSO	dimethyl sulfoxide
PMA	phorbol 12-myristate-13-acetate
SRBC	sheep red blood cells
TNP-OVA	trinitrophenyl-ovalbumin
FBS	fetal bovine serum
HRP	horseradish peroxidase
FITC	fluorescein
PE	phycoerythrin
PFC	Plaque-forming cell (s)
ELISA	enzyme-linked immunosorbent assay
ELISPOTS	enzyme-linked immunospots assay
FACS	Fluorescence-activated cell sorting
Mab	monoclonal antibody
GS-1	*Griffonia simplicifolia-1*
CFA	complete Freund's adjuvant
IFA	incomplete Freund's adjuvant
i.p.	intraperitoneal
i.v.	intravenous
MEM	minimum essential medium
HBSS	Hanks balanced salts solution
IL-1β, -2, -4, -5, -10	interleukin-1β, -2, -4, -5, -10
INF-γ	interferon-γ

INTRODUCTION

I. Molecular Structure and genetic properties of IgD

Since its discovery, the biologic role of immunoglobulin D (IgD) has remained enigmatic despite numerous experimental attempts to define its function. Its biological function is generally described in negative rather than positive terms: it is not secreted following antigenic or mitogenic stimulation of IgD^+ B cells, it does not appear to neutralize antigen, and it does not fix complement. These negative functional properties have been used to support the view that IgD is primarily a cell-surface antigen receptor (Pernis, 1977).

IgD is a predominant surface immunoglobulin (Ig) expressed in high density on B lymphocytes (Vitetta, 1974; Van Boxel et. al., 1992). The majority of mature, resting B lymphocytes co-express membrane IgD along with IgM. In both human and murine systems, the genes encoding the constant region of the μ (Cμ) and δ (Cδ) chains reside in a single transcription unit and can be expressed simultaneously, by alternative splicing of the V region exon to the first exon of Cμ or the Cδ gene which generate both μ mRNA and δ mRNA.(Van Boxel et. al., 1992) (Maki et. al., 1981; White et. al., 1985; Milstein et. al., 1984; Word et. al., 1989). The earliest Ig-producing B lineage cell, the pre-B cell, produces μ heavy (H) chains in the absence of light (L) chain synthesis or L chain gene rearrangement and be associated with surrogate light chain (λ5vpreB) (Kincade, 1981; Cooper and Burrows, 1989). As differentiation continues, rearrangement and expression

of a conventional L chain gene occurs, leading to expression of IgM monomers as antigen receptors on the surface of the newly formed B cells (Fu et. al., 1974; Vitetta et. al., Kearney et. al., 1977). Newly formed immature B cells in the bone marrow or fetal liver express IgM but not IgD on their surface, whereas the vast majority of mature peripheral B cells of an adult mouse are characterized by surface expression of IgM and IgD (Vitetta and Uhr, 1977; Goding et. al., 1977). IgD is a major B cell antigen receptor coexpressed with IgM on the surface of mature B lymphocytes in mouse, human, and a variety of other species (Martin and Leslie, 1977; Abney and Parkhouse, 1974; Melcher et. al., 1974; Ruddick and Leslie; 1977; Eskinazi et. al., 1979; Wilder et. al., 1979;). Once acquired as a surface Ig isotype, IgD expression exceeds IgM by about 10-fold in fully mature B cells (Havran et. al., 1984). During ontogeny, the first $\mu^+\delta^+$ B cells appear about day 4 after birth in the mouse, and the frequency of these cells increase to adult levels by 4-6 weeks of age (Ruddick and Leslie, 1977; Eskinazi et. al., 1979; Wilder et. al., 1979). IgM as well as IgD can mediate B cell activation (Sieckmann et. al., 1982), and it seems that both receptors can transduce signals by the same mechanism (Cambier et. al., 1987). Expression of IgD is down-regulated during primary immune response following antigenic stimulation of B cells (Bourgois et. al., 1977; Monroe et. al., 1983). The down-regulation of IgD is accompanied by an increase in IgM expression, and subsequent secretion of this isotype in the early primary response (Roes and Rajewsky, 1989; Kocks and Rajewsky, 1993).

IgD has a molecular weight of 175 kDa (Paul, 1989). Similar to other immunoglobulin classes, it consists of two identical heavy chains and two identical light

chains (Rowe and Fahey, 1965). However, IgD is more heavily glycosylated than any of the other Ig isotypes with 14% of its molecular mass attributable to carbohydrates (Leslie and Martin, 1977). Because of its long hinge region, which is susceptible to proteolysis, IgD is an unstable molecule with a short half-life of 2-3 days in comparison with 23,6, and 5 days for IgG, IgA, and IgM, respectively (Leslie and Martin, 1977).

The role of IgD as an antigen receptor is clearly established and evidence has shown that this Ig isotype is involved in antigen-mediated recruitment of B cells. Other studies have suggested that IgD may play an important role in the transition from a stage of susceptibility to tolerance induction, to one of responsiveness (Noaaal and Pike, 1975; Metcalf and Klinman, 1976, Cambier et. al., 1977; Scott et. al., 1977; Vitetta and Uhr, 1975) although contradictory results have also been reported (Layton et. al., 1978; Zitron et. al., 1977; Cambier et. al., 1978; Jacobson et. al., 1982; Lislie and Cuchens, 1982; Bazian et. al., 1978; Mosier et. al., 1977). Recent studies by Sandel and Monroe have challenged this hypothesis based upon an analysis of the mechanisms that determine how B cells are negatively selected (Sandel and Monroe, 1999). They demonstrated that negative selection of immature B cells by receptor editing or deletion is determined by the site of antigen encounter. Unlike the peripheral environment where transitional immature B cells (whose phenotype is intermediately high with respect to IgD) are deleted, the bone marrow microenvironment provides signals that block antigen-induced deletion and promote recombination activation gene (*RAG*) reinduction. The latter leads to receptor editing and results in the generation of fully mature B cells that express high levels of IgD and intermediate levels of IgM. Thus, they propose that the mechanism of tolerance induction of immature B cells determined, not by developmental stage, but by

the environment in which immature B cells first encounter antigen. IgD expression has also been suggested to be necessary for the propagation of B cell memory (Zan-Bar et. al., 1979) but, again, contradictory results have also been reported (Black et. al., 1980; Herzenberg et. al., 1980).

II. Identification of IgD-receptors and their role in immune regulation.

Our laboratory was the first to demonstrate that in addition to recognition and binding to antigen, IgD also serves as a ligand for IgD-specific receptors (IgD-R) expressed on $CD4^+$ T cells (Coico et. al., 1985a; Coico et. al., 1985c; Coico et. al., 1988a; Coico et. al., 1988b; Coico et. al., 1990). We have called such cells with up-regulated IgD-R "Tδ cells". In the murine system, exposure to multimeric IgD *in vivo and in vitro* significantly up-regulates the number of Tδ cells (Yang and Coico, 1996). The search for IgD-R^+ cells began with studies of IgD plasmacytoma-bearing mice. (Finkelman et. al., 1981). BALB/c mice injected with IgD-containing ascites or with isolated IgD protein showed significantly enhanced humoral immune responses *in vivo* (Xue et. al., 1984). Experiments exploring the cellular basis for this effect led to the identification of IgD-R expressed on $CD4^+$ T cells and demonstrated that the immunoaugmenting effects of IgD-pretreatment could be adoptively transferred by this subpopulation of T cells (Coico et. al., 1985b; Coico et. al., 1985c). In normal control mice, IgD-R's are present on a small percentage (<5%) of T lymphocytes in spleens and lymph nodes. Injection of oligomeric IgD results in rapid increase in the number of $CD4^+$ IgD-R^+ T cells (~30%).

Other stimuli shown to upregulate IgD-R expression on T cells include: antigenic stimulation, exposure to anti-CD3; exposure to phorbol 12-myristate 13-acetate (PMA) + ionomycin, exposure to IgD-containing immune complexes, exposure to certain cytokines including IL-2 and IL-4, and co-culture of T cells with fixed B cells expressing cross-linked membrane IgD (Coico et, al., 1988b; Coico et. al., 1985a; Coico et. al., 1985c; Coico et. al., 1987). IgD-R have also been identified on human T cells although it appears that both $CD4^+$ and $CD8^+$ cells are capable of expressing these receptors (Coico et. al., 1990) (Figure A).

Unlike receptors for other Ig classes, which are specific for the Fc regions of the Ig molecule and therefore called Fc receptors (Ravetch and Kinet, 1991), IgD-R present an exception to this rule (Swenson and Thorbecke, 1997). Studies carried out by Tamma et al. and Amin et al. have shown that murine IgD-R recognizes the Cδ1 or Cδ3 region of IgD (Tamma et. al., 1991; Amin et. al., 1991). The binding specificity of IgD-R to IgD appears to be linked to IgD-associated carbohydrates because deglycosylated IgD fails to bind to these receptors (Amin, et. al., 1991). Moreover, N-acetylgalactosamine or N-linked glycans isolated from IgD blocks the binding of IgD-R to IgD-coated target cells, suggesting that murine IgD-R have lectin-like properties (Tamma et. al., 1991; Amin et. al., 1991; Tamma and Coico, 1992; Swenson et. al., 1998; Swenson et. al., 19993). Therefore, upregulation of IgD-R on $CD4^+$ T cells may facilitate their interaction with specific carbohydrate moieties uniquely associated with membrane IgD on B cells.

Thus, our approach to the study of the physiology of IgD has been based upon our observation that injection of dimeric or oligomeric IgD causes upregulation of IgD-

receptor R^+ T cells) and therefore enhanced ability of such cells (Tδ cells) to respond to antigen-presenting B cells (Swenson and Thorbecke, 1997). The identification of IgD-R expressed on T cells following their exposure to oligomeric IgD has suggested a role for these receptors in T-B interactions which might explain the immunoregulatory properties of Tδ cells. This hypothesis is supported by several lines of evidence including the finding that treatment with oligomeric IgD enhanced primary and secondary Ab responses to antigens in normal but not athymic mice (Xue et. al., 1984; Coico et. al., 1988; Coico et. al., 1985a; Coico et. al., 1985c; Coico et. al., 1987). Enhanced production of all Ig isotypes is seen with the exception of IgD (Swenson, et, al., 1988). In addition, treatment with oligomeric IgD also results in a significant increase in the percentage of follicles with germinal centers (Swenson et. al., 1988).

It is clear that cross-linking of IgD-R on $CD4^+$ T cells is one mechanism responsible for induction of IgD-R expression on T cells. Antigen and oligomeric IgD are almost equally effective in inducing up-regulation of IgD-R in $IgD^{+/+}$ mice. Monomeric IgD blocks the up-regulation of IgD-R and also prevents the immunoaugmention caused by oligomeric IgD *in vivo* and *in vitro*. Moreover, monomeric IgD also inhibits the Ag-induced up-regulation of IgD-R on T cells in vivo, although it has no effect on the primary response, it does cause a delay in the priming for secondary response (Swenson et. al., 1995). These findings strongly suggest that cross-linking of IgD-R by oligomeric IgD or anti-IgD immune complex is critical for IgD-R up-regulation on T cells and the immunoaugmenting effect of Tδ in normal mice is mediated through interaction with IgD on the membrane of B cells (Coico et. al., 1990;

Coico et. al., 1988; Coico et. al., 1988; Swenson et. al., 1995). Studies of the effects of IgD-R up-regulation on antibody responses utilizing $IgD^{-/-}$ mice supports this hypothesis. Although injection of oligomeric IgD, but not antigen, leads to the up-regulation of IgD-R on T cells in $IgD^{-/-}$ mice, neither the augmenting effect of oligomeric IgD nor the inhibitory effect of monomeric IgD on antibody response is observed in these mice (Swenson et. al., 1995). In addition, in aged mice, up-regulation of IgD-R on T cells following exposure to IgD has never been detected, and, as predicted by this hypothesis, the immunoaugmenting effects of IgD are also absent (Coico et. al., 1987; Swenson et. al., 1988).

IgD-deficient mice ($IgD^{-/-}$) have also been used by others to assess the biologic function of IgD (Jurgen and Rajewsky, 1993). These studies have demonstrated that $IgD^{-/-}$ mice respond to T-independent and T-dependent (TD) antigens although affinity maturation and germinal center development in early TD primary responses is delayed, suggesting a particular role for IgD in the recruitment of B cells into the germinal centers (Nitschke et. al., 1993; Swenson et. al., 1995). Given the obligatory role of T cells in the generation of B cell memory, (Jacobson, et. al., 1974) our data correlating increased germinal center development with the induction of IgD-R^+ T cells (Swenson et. al., 1988) together with knowledge that the precursors of germinal centers (Linton et. al., 1992) and memory B cells (Linton et. al., 1989) reside in the $J11D^{low}$,IgD^{high} B cell subpopulation, suggest that interaction between membrane IgD on B cells and IgD-R on T cells may facilitate germinal center development.

III. Helper T cell-B cell interactions and the role of different costimulatory molecules in

T and B cell activation.

T cell cytokine production requires activation and clonal expansion of responding T cells – a phenomenon which is dependent on ligation of the TCR as well as other costimulatory molecules expressed on the T cell. T cell activation involves a number of signaling steps which lead to cytokine production beginning with the specific binding of antigen (itself bound to MHC class II molecules). Activation of small, resting T cells requires two distinct signals: one signal is generated from the interaction between TCR/CD3-CD4/CD8 and Ag-MHC; the second signal is dependent on interactions between key co-stimulatory molecules. B cells serve as antigen presenting cells for T cells and T-B cell interactions result in antibody responses. A direct signal through binding of antigen to membrane Ig, followed by antigen processing and presentation to T cells can enhance T-dependent B cell activation. Antigen recognition by T cell is sequential rather than simulataneous: first the B cell binds antigen with its antigen receptor menbrane Ig and internalizes and degrades the antigen; then it presents peptides from the antigen on the cell surface bound to classII MHC molecules; finally the T cell recognizes the processed antigen on B cell surface through the antigen-specific T cell receptor. As a result of helper T cell recognition of antigen on the B cell surface, the T cell becomes activated and in turn activates the B cell. B cells then obtain help from T cells in antibody response. T cell help is provided by two functional components: 1) they secrete cytokines which act as growth and differentiation factors for B cells, and 2) they express costimulatory molecules following activation which ligate other specific costimulatory molecules expressed by B cells. Examples of well-characterized T cell and

B cell costimulatory pairs include CD28-B7 and CD40-CD40L, which facilitate T and B cells interaction during antigen presentation and enable B cells to respond to cytokines. LFA-ICAM-1 is another important signaling/adhesion ligand pairs. A rapidly increased affinity between the adhesion molecules LAF-1 and ICAM-1 has been shown to be required for T cell-B cell activation (Parker, 1993; Dustin and Springer, 1989).

In addition to the CD28/B7, CD40/CD40L and LFA-1/ICAM-1 interaction, a number of costimulatory molecules have been found to be involved in T and B cell collaboration which include $CD45R_0$/CD22 (Stamenkovic et. al., 1991) and CTLA-4/B7 (Linsley and Ledbetter, 1993). These molecules are known to either act as adhesion/signaling accessory molecules or as costimulatory molecules to modify the T cell response to antigen recognition, and to deliver essential, activation growth signals to the B cell (Parker, 1993) (Figure B).

Perhaps the most important of the costimulatory molecules expressed by T cells (and some other cells as well) is CD28, a disulfide-linked homodimeric glycoprotein, which generates an important costimulatory signal to T cells triggered by its receptors, B7-1 and /or B7-2, expressed on APC (Allison 1994; Freedman et. al., 1987; Freedman et. al., 1991; Azuma et al., 1993; Linsley and Ledbetter, 1993). CD28-mediated costimulatory signals are critically important for the initiation of cytokine gene transcription and regulation of cytokine mRNA stability (Blustone, 1995; Green and Thompson, 1994), clonal expansion of Ag-specific T cells, and prevention of T cell unresponsiveness or anergy to repeated stimulation (Shi et. al., 1995; Boise et. al., 1995). Blocking the CD28- B7 interaction with soluble CTLA-4 Ig, blocks the induction of cytokine synthesis (Seder et al., 1994; Lenschow et al., 1992). Moreover, resting B cells

do not express B7-1 or B7-2 molecules are thought to induce tolerance in responding T cells, and activated B cells which express B-7 molecules are very effective at inducing T cells activation (Freedman et al., 1987; Freeman et al., 1993; Gajewski et al., 1994). CD28/B7 interactions also play a role in the early differentiation of Th subsets (King et. al., 1995; Webb and Feldmann, 1995).

An additional T cell membrane protein is a CD28 homologe, namely, CTLA-4 (Harper et. al.,1991). This T cell-specific marker is transiently expressed on the cell surface only after T cell activation as a disulfide-linked homodimer(Linsley et. al., 1995). CTLA-4 is also the high avidity receptor for B7-1 (CD80) and B7-2 (CD86) (Linsley et. al., 1991), but unlike CD28 that sends positive T cell activation and proliferation signal, it sends a negative signal to the T cell (Boise et. al., 1995; Krummel et. al., 1995; Waterhouse et. al., 1995; Tivol et. al., 1995). The interaction of CTLA-4 with its ligands may either signal the T cell to stop proliferating and die or deprive the cells of necessary positive signals delivered via the CD28 molecules (Bluestone, 1995). Strong evidence for the role of CTLA-4 in negative regulation of T cell activation is provided by the severe lymphoproliferative phenotype of CTLA-$4^{-/-}$ mice (Tivol et. al., 1995). Thus, CTLA-4 may be an important T cell down-regulatory molecule. Since CTLA-4 has a much higher affinity for both B7-1 and B7-2 ligands, control of the immune response may depend on a competition between CD28 and CTLA-4 on the activated T cell for B7-1 and B7-2 on antigen presenting cells (APCs) (Bluestone, 1995).

Another costimulatory molecule, CD40L, has been shown to be induced after T cell activation. The interaction of CD40 and CD40L can result in B cell proliferation, differentiation, Ig secretion, and Ig isotype class switching (Banchereau et. al., 1991),

preventing germinal B cells apoptosis (MacLennan et.al., 1992). CD40-CD40L interaction is critical for T-dependent Ab response (Foy et. al., 1993). Blockage of CD40 and CD40L interaction can cause inhibition of both primary and secondary antibody responses to T-dependent antigens *in vivo* (Korthauer et. al., 1993). Human X-linked hyper IgM syndrome is usually caused by mutations in CD40L (Disanto et. al., 1993; Aruffo et. al., 1993; Fuleihan et. al., 1993; Allen et. al., 1993). Similarly, mice with targeted defects in expression of CD40L (Xu et. al., 1994; Renshaw et. al., 1994) or CD40 (Kawabe et. al., 1994) fail to produce Ag specific IgG1 response, do not develop germinal centers, and are unable to develop memory B cells in response to T-dependent antigens (Linna et.al., 1995). Thus, CD40 and CD40L both plays a critical role in T cell-dependent B cell activation, proliferation and differentiation. They are also important for accessary cell mobilization events which are needed for this process of B cell activation. It is noteworthy that a major hypothesis of the current investigation is that IgD and IgD-R represent a costimulatory pair involved in T cell and B cell activation. As will be discussed, we have explored this possibility by examining various well-characterized activation events associated with T cell upregulation of IgD-R using a cross-linking model that mimics the effects of interactions between T cells and IgD^+ B cells. Among the experimental approaches used to assess the functional parameters affected by IgD-R crosslinking are effects on the signal transduction machinery and early activation markers associated with T cell activation.

CD45 is the major protein tyrosine phosphatase of lymphocytes and probably plays a key role in signalling in both T and B cells through their antigen receptors. The $CD45R_0$ is the dominant CD45 isoform in murine T helper lines. They probably

costimulate or regulate helper T cell activation (Rogers et. al., 1992).

CD69 is a very early activation marker of T lymphocytes. It is a cell surface glycoprotein that can only be detected following T cell activation (Hamann et. al., 1993). Crosslinking of CD69 was able to induce T cells proliferation (Cebrian et. al., 1988). Sequence analysis has shown that CD69 is a type II integral membrane protein with a C-type lectin domain, and that phosphorylated serine residues within its intracellular domain may play a role in transmembrane signaling (Hamann et. al., 1993; Worg et. al., 1991; Giorda et. al., 1990). It is worth mentioning at this point that in light of the coincidental early upregulation of CD69 and of IgD-R following T cell activation and the fact that the natural ligand for CD69 has not yet been identified, it was of interest to examine whether CD69 itself is the IgD-R or has homology to IgD-R in the present study.

In summary, T-B cell collaboration during antigen presentation can lead to upregulation of several costimulatory molecules. The functions of these molecules is likely to be complex and redundant. Perhaps the most important pairs of costimulatory molecules known to be involved in T-B interaction are B7/CD28 and CD40/CD40L (Linna et. al., 1995; Aruffo et. al., 1993; Fuleihan et. al., 1993; Allen et. al., 1993). Our previous studies have demonstrated that interaction of IgD and IgD-R significantly enhances humoral immune responses (Coico et. al., 1984; Tamma et. al., 1991). Signaling pathways associated with this regulation are still unclear. In the present study we show that transient expression of CD28 occurs following cross-linking of IgD-R on $CD4^+$ T cells. The results indicate that interaction between IgD-R on $CD4^+$ T cells with IgD on B cells may play a role in regulation of immune response through the CD28-

mediated signaling process.

IV. Correlating immune responses and CD4$^+$ T cell subset.

Effector functions in the immune system are carried out by a variety of cell types. At the simplest level, this can be summarized with respect to the humoral immune response by stating that B lymphocytes produce antibody and T lymphocytes provide help for B cells (Claman and Chaperon, 1966; Mitchell and Miller, 1968). The T cell population is further divided into CD4$^+$ T helper cells and CD8$^+$ T cytotoxic cells (Cantor and Boyse, 1975). Studies with mouse CD4$^+$ T cell clones led to the finding that CD4$^+$ T cell clones can be divided into two distinct subsets with distinct functional abilities (Mosmann et. al., 1986). Based on their functional capabilities and the pattern of cytokine production, these clones could be subdivided into those that participate in cell-mediated immune responses such as delayed-type hypersensitivity (DTH) and macrophage activation (Th1 subset), and those that induce B cells to secrete antibodies (Th2 subset). Cytokines such as IL-2, INF-γ, and TNF-β are secreted by the Th1 subset which plays a key role in inflammation and cell-mediated immune responses. Cytokines such as IL-4, IL-5, IL-6, IL-10 and IL-13 produced by the Th2 subset help B cells proliferate and differentiate and play an important role in humoral immune responses (Mosmann and Coffman, 1989; Maggi e t a l., 1992; Romagnani, 1991; Romagnani, 1995). A third CD4$^+$ T cell subset, called Th0 cells, produces a mixture of the two cytokine patterns (Firestein GS et. al., 1989; Paliard X et. al., 1988). Analyses using

approaches of *in situ* mRNA hybridization and intracellular protein staining in which cytokines can be detected in individual cells have shown that overlapping cytokine profiles are due to the presence of mixed populations of $CD4^+$ T cell subsets (Th0) (Carding SR et. al., 1989; Openshaw P et. al., 1995). The different classes of Ig secrection are influenced by different cytokine productions, which activate transcription of Ig constant region genes in their germline configurations (Severinson and Stavnezer, 1990). The Th1 helper cells preferentially induce IgG2a, while Th2 helper cells induce IgG1 and IgE secrection.

Th1/Th2 differentiation has been shown to be influenced by many different factors, such as cytokines, the APC population used to stimulate T cells, and costimulatory molecules. IL-10 promote the development of Th2 cells and suppresses Th1 cells. In contrast, INF-γ and IL-12 promote Th1 cells differentiation and prevent Th2 cell growth (Seder and Paul, 1994). Different costimulatory molecules on APC may provide the precursor Th cell with specific initial activation signal and influence Th1/Th2 differentiation (Constant and Bottomly, 1997). Costimulatory molecules, such as the CD28/B7 ligand pair, might provide an alternative mechanism for regulating Th1/Th2 immunity. Blocking CD28/B7 interactions greatly reduced IL-2 production and proliferation in Th1 clones suggesting a role for CD28 in the early differentiation of Th subsets (Mcknight et. al., 1994; King et. al., 1995; Webb and Feldmann, 1995). Other studies found that blocking CD28/B7 interactions with CTLA4-Ig selectively abolishes IL-4 production (Seder et. al., 1994). In addition, $CD28^{-/-}$ mice have been shown to have defects in Th2-dependent antibody responses to lymphocytic choriomeningitis virus

(LCMV), while anti-LCMV DTH and cytolytic response are normal (Shahinian et. al., 1993).

Studies have shown that elevated serum IgD in humans can result in impressive cytokine production in PBMC in vitro (Drenth et. al., 1994; Drenth et. al., 1995). We suggest that IgD and IgD-R may be part of the redundancy costimulatory molecules in regulating Th1 or Th2 response. In current study, we analyzed the cytokine profiles of T cells following cross-linking IgD-R. We further explored whether antigen-presenting B cells expressing IgD preferentially helped to activate Th1 or Th2 helper cells.

V. IgD-R-Mediated Signal Transduction.

Cellular receptors for Fc domain of immunoglobulins (FcRs) are known to be widely distributed on cells of immune system (Ravetch and Kinet, 1991; Ravetch, 1994). FcR show specificity for individual Ig isotypes and mediate effector responses when cross-linked *in vitro* and *in vivo* (Ravetch and Kinet, 1991; Ravetch, 1994). Some of the effector functions like antibody-dependent cytotoxicity, mast cell degranulation, phagocytosis, lymphocyte proliferation, and antibody secretion have been shown to be mediated by Fc receptors. Because we have observed that that IgD-R bind the Cδ1 and Cδ3 heavy chain domains of IgD, we have not designated these receptor as FcδR (Tamma et. al., 1991; Amin et. al., 1991; Tamma and Coico, 1992; Swenson et. al., 1998). Nevertheless, their specificity for IgD places them in the family of well-known Ig-binding receptors known to function within the immune system (Raghavan and

Bjorkman, 1997; Adachi and Ishizaka, 1988). Most human and murine FcRs are members of the immunoglobulin superfamily (IgSF); others belong to the lectin family (e.g. FcεR, IgD-R) (Tamma et. al., 1991; Amin et. al., 1991; Ravetch and Kinet, 1991). Many FcRs trigger cellular responses using the same signal transduction pathways as antigen receptors. In order to mediate signal transduction, FcRs must be aggregated or crosslinked at the cell surface (e.g. using antibodies or multivalent antigens). FcRs capable of triggering cell activation possess one or more intracytoplasmic activation motifs which resemble those of the B cell receptor (BCR) and T cell receptor (TCR) signal transduction subunits; these motifs composed by a twice repeated YxxL sequence flanking seven variable residues designated as ITAMs (immunoreceptor tyrosine-based activation motifs) (Cambier et. al., 1994). Phosphorylation of the tyrosines within these motifs is necessary for eliciting the functions associated with these receptors (Neuberger and Rajewsky, 1981). An early intracellular event following aggregation of FcR induce the phosphorylation of tyrosine residues within ITAMs, which activate sequentially Src family tyrosine kinases and Syk family tyrosine kinases that connect transduced signals to common activation pathways shared with other receptors. Once activated by Syk , PLC-γ1 generates two metabolites called second messenger by hydrolysis of phosphatidylinositol 4,5-bisphosphate. Hydrolysis of phosphatidylinositol 4,5-bisphosphate produces diacylglycerol which activates PKC, and inositide 1,4,5-triphosphate which triggers mobilization of intracellular calcium stores and influx of extracellular calcium. Some signals triggered by FcR reach the nucleus via the *ras* pathway. *Ras* phosphorylates *raf*, which phoshporylates Mek kinase, which eventually

phosphorylates the Map kinases. Once in the nucleus, Map kinase activates transcription factors (AP-1), leading to DNA binding and transcription of various genes (Cheng et. al., 1995; Chan et. al., 1992; Motto, et. al., 1996; Wovdrow et. al., 1993; John J. O'Shea et. al., 1996) (Figure C).

IgD-R$^+$ T cells display several enhanced functional properties as compared with control CD4$^+$ T cells. The most striking function of IgD-R$^+$ T cells concerns their enhanced ability to help B cells respond to antigen (Xue et. al., 1984; Coico et. al., 1988b). As noted earlier, studies have shown a correlation between upregulation of IgD-R expression by IgD-R$^+$ T cells and increased ability of such cells to transfer helper activity for antibody responses (Xue et. al., 1984). Upregulation of IgD-R facilitates cognate T-B interactions (most likely through ligation of carbohydrate moieties associated with IgD itself), resulting in increased antibody responses and clonal expansion of antigen-specific T cells (Yang and Coico, 1996; Wu and Coico, 1999). These findings indicate that B cell membrane IgD serves not only as a receptor for antigen together with membrane IgM, but also as the ligand for IgD-R expressed on the CD4$^+$ T cells. This effect on antibody responses is absent in IgD$^{-/-}$ mice despite the ability of T cells from such mice to express IgD-R (Swenson et. al., 1995). Taken together these studies suggest that IgD-R may mediate bidirectional signaling during cognate T-B interactions. In the current study, we analyzed the intracellular signaling events in T cells mediated by IgD-R cross-linking. Monomeric forms of IgD added to such cultures block the functional activities of IgD-R$^+$ T cells whereas other Ig isotypes have no effects (Yang and Coico, 1996).

VI. Expression of Fas antigen/apoptosis.

Fas (CD95) antigen is a 36 kDa transmembrane glycoprotein which belongs to the nerve growth factor (NGF)/tumor necrosis factor (TNF) receptor family of surface molecules (Yonehara et. al., 1989). Several studies have suggested that Fas antigen may mediate apoptosis (Trauth et. al., 1989; Ito, et, al., 1991; Miyawaki et. al., 1992). Murine cell lines transfected with the human Fas antigen cDNA have been shown to be effectively killed by anti-Fas monoclonal antibody through apoptotic cell death (Ito et. al., 1991). In response to TCR engagement, CD95 has been shown to be upregulated followed by CD95L upregulation (Suda et. al., 1993; Lynch et. al., 1994; Brunner et. al., 1995; Dhein et. al., 1995; Ju et. al., 1995; Van parijs and Abbas, 1996). Binding of CD95 to CD95L triggers clonal deletion (by apoptosis) of the cycling T lymphocytes (Suda et. al., 1993; Lynch et. al., 1994; Brunner et. al., 1995; Dhein et. al., 1995; Ju et. al., 1995; Van parijs and Abbas, 1996)

Given the functional consequences of upregulation of IgD-R on $CD4^+$ T cells following crosslinking of basal receptors with oligomeric IgD and the fact that inhibitors of protein tyrosine kinases (PTK) block this upregulation (Amin et. al. 1993; Swenson et. al. 1993), we also investigated the ability of IgD-R to transmit intracellular signals associated with T cell activation. These studies were carried out together with experiments designed to assess the effects of IgD-R crosslinking on apoptosis in $CD4^+$ T cells.

VII. Objectives

The studies carried out under this thesis project had several objectives:

1) To assess the role of antigen-presenting B cells on IgD-R$^+$ T cell activation. This was investigated, in part, by comparing B cells from IgD$^{+/+}$, IgD$^{+/-}$ and IgD$^{-/-}$ mice to determine whether IgD expression is a prerequisite for functional activity of IgD-R$^+$ T cells. Since monomeric IgD has been shown to block the development of germinal centers, implicating a role for the IgD-R in the development of these structures (Swenson et. al., 1995), we investigated the inhibitory effects of monomeric IgD on IgD-R-IgD interactions and resulting T cell-B cell responses.

2) To characterize the immunoaugmenting effects of IgD-R$^+$ T cells on B cell responses. This was accomplished using B cells isolated from IgD$^{+/+}$, IgD$^{+/-}$ and IgD$^{-/-}$ mice.

3) To determine whether ligation of IgD and IgD-R promote T-B interactions through a mechanism that involves bi-directional signals. Studies reported earlier and further explored in the present study have made it clear that such ligation delivers signals to B cells that result in enhanced antibody responses, Ig class-switching and germinal center production. In the present study, we asked whether signals are also delivered to the T cell following IgD-IgD-R ligation. This was investigated by performing experiments that measured several T cell

functional parameters such as cytokines. We analyzed cytokine profiles, both at mRNA level and protein level following in vivo and in vitro stimulation by IgD-R cross-linking.

4) To assess whether the bi-directional signals result in up-regulation of other co-stimulatory molecules.

5) To analyze the intercellular signaling events associated with IgD-R-IgD interaction in helper T cells.

6) To characterize the molecular properties of IgD-R isolated from IgD-R-expressing T hybridoma cells.

Collectively, our studies lead us to conclude that IgD-R are, indeed, involved in bi-directional signaling of B and T cells. As will be shown in the results section, evidence from our cytokine studies also indicate that when B cells act as APC for T cells expressing IgD-R, this may skew responses towards the Th2 phenotype. This observation is consistent with the functional properties of IgD-R$^+$ T cells which are known to significantly enhance antibody responses. Finally, studies of Fas antigen regulation and expression associated with upregulation of IgD-R suggest that ligation of IgD-R may protect T cells from undergoing apoptosis following their activation by IgD-expressing B cells.

MATERIALS AND METHODS

Mice

Four to eight week-old male and female BALB/C and C57B1/6 mice were purchased from Charles River Laboratories (Wilmington,MA). Male and female $IgD^{-/-}$ mice (IgD-KO mice) generated by gene targeting on a 129 x C57B1/6 background, were kindly provided by Dr.Klaus Rajewsky (University of Cologne, Germany) (Rajewsky,K 1993). These mice contain B cells lacking IgD and were bred in CUNY Medical School animal facility, as were $IgD^{+/-}$ [IgD-KO x C57B/6]F1 control mice. Lack of cell surface expression of IgD was verified by flow cytometric fluorescence analysis.

Cell lines

Mouse $CD4^+$, $IgD\text{-}R^+$ T hybridoma cell lines (C5-E1 and 1D1-E1) were kindly provided by Dr.Vincent Tsiagbe (NYU School of Medicine) and were maintained in EMEM (GIBCO-BRL, Grand island, NY) medium containing 10% fetal bovine serum (FBS) and 10% of a nutrient cocktail prepared by combining the following components: 7.5g D-glucose, 75 ml 50x MEM essential amino acids, 37.6 ml 100x MEM non-essential amino acids, 100 ml 10x L-glutamine and 8.5g Na_2HCO_3, 100 ml 10x penicillin/streptomycin, 500 mg gentamicin and 8.5 µl 2-mercaptoethanol; the volume of the cocktail was then brought up to 1000 ml with EMEM medium and the pH was adjusted to 7.0.

A hamster T hybridoma cell line (H1.2 F3) that secrets anti-mouse CD69 monoclonal antibody (Mab) was kindly provided by Dr.Ethan Shevach (NIAID, NIH).

The cell line was maintained in RPMI1640 medium (GIBCO-BRL, Grand island, N.Y) containing 10% FBS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 pq/ml recombinant IL-1β.

Antibodies and Reagents

Normal goat Ig, goat anti-mouse (GAM) IgG/IgA/IgM (GAM Ig) and GAM IgM were obtained from Jackson Immunoreseach Laboratories, Inc., (West Grove, PA). Goat anti-mouse IgD (GAM IgD) was purchased from Nordic Immunologicals, Inc., (Capistrano Beach,CA). Sheep red blood cells (SRBC) were obtained from Colorado Serum Co. (Denver). IgD was biotinylated as described below according to the method of Lisanti and Sargiacomo (1995). PE-conjugated hamster anti-mouse CD28 (CD28-PE), PE-conjugated hamster anti-mouse CD40L (CD40L-PE), PE-conjugated rat anti-CD4 (CD4-PE), FITC-conjugated rat anti-CD4 (CD4-FITC), FITC-conjugated armenian hamster anti-mouse CD3e (CD3-FITC), FITC-conjugated B220 (B220-FITC), PE-conjugated hamster Ig, PE-conjugated Rat Ig, avidin-FITC , rat anti-mouse CD16/CD32 (2.4G2) and hamster anti-mouse CD69 monoclonal antibody, FITC-conjugated murine Fas (Jo2 clone) and PE-conjugated Fas-ligand (MFL3 clone) antibody, Annexin V-FITC and Propidium Iodide (PI) were purchased from Pharmingen (San Diego, CA). Rat anti-mouse IgD and FITC-conjugated rat anti-mouse IgD were purchased from Southern Biotechnology Associates, Inc., (Birmingham, AL). Horseradish peroxidase (HRP)-conjugate goat anti-rat Ig and HRP-conjugated streptavidin were purchased from Jackson Immunoresearch Laboratories, Inc (West Grove, PA). Hamster anti-mouse CD3-ε Mab was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA.).

For intracellular staining, FITC-conjugated rat anti-mouse INF-γ and FITC-conjugated rat anti-mouse IL-2, PE-conjugated rat anti-mouse IL-4 and PE-conjugated rat anti- mouse IL-5, PE or FITC-conjugated rat Ig, and cell permeabilization kits were purchased from Caltag Laboratories (Burlingame, CA.).

Purification of IgD

TEPC-1017 myeloma cells secreting oligomeric IgD were maintained by i.p. transfered in pristine-primed BALB/C mice (Finkelman, et al., 1981). IgD was purified by affinty chromatography by utilizing *Griffonia simplicifolia*-1 (GS-1) -Sepharose (EY Laboratories, Inc., San Mateo, CA) (Coico and Amin, 1997). Initially, IgD-containing ascites fluid from mice bearing plasmacytomas was harvested, mixed 1:9 with ice-cold PBS-CA buffer, and centrifuged 30 min. at 13,000 rpm, 4°C. Supernatant was collected and treated with an equal volume of delipidating agent (Clinetics, Tustin, CA), vortexd until it became a homogenous mixture, then centrifuged for 5 minutes at 3000 rpm at 4°C. The clarified aqueous phase was then collected for column chromatography. GS-1-Sepharose columns were prepared according to Oppenheim and Amin (1990) and washed successively with 10 column volumes of each of the following ice-cold buffers: PBS-CA, PBS-CA-GAL, and PBS. Cleared, delipidated ascites was applied to the column. The column was washed with ice-cold PBS-CA until UV-monitored spectrophotometric recordings approached baseline value (washing fractions were saved for measuring the A_{280} values spectrophotometrically). IgD was eluted by washing the column with ice-cold PBS-CA-GAL. Eluted material was dialyzed against 1 liter ice-cold PBS, pH 7.3, at 4°C (dialysis buffer was changed three times). IgD concentration was assessed

spectrophotometrically by measuring A_{280}. The purity of IgD was analyzed by ELISA and using 10% SDS-polyacrylamide gels (Sigma, St. Louis, MO) under reducing conditions. IgD was stored at ≥1mg/ml in ice-cold PBS, pH 7.3, at -70°C.

The B cell hybridoma lines, KWD9, KWD8, KWD1, B1.8δ which secrete monomeric forms of IgD, were kindly provided by Dr.Fred Finkelman (University of Cincinnati Medical Center) and maintained either in BALB/c mice as ascites (using the same approach described above for TEPC-1017 plasmacytoma cells) or in cell culture.

Biotinylation of IgD: 50 mg sulfo-NHS-biotin (Sigma, St. Louis, MO) was dissolved in 250 µl dimethyl sulfoxide (DMSO) by vortexing vigorously to make 200 mg/ml stock solution. Purified IgD was thawed and added to sulfo-NHS-biotin stock solution to a final concentration 0.5-1 mg/ml and incubated on ice for 30-60 minutes. Two hundred µl of serum free culture media RPMI1640 (Gibco-BRL) was then added to quench unconjugated biotin material. The conjugated protein solution was dialyzed overnight against PBS at 4°C. Aliquots were stored at -20°C for up to 6 months.

Quantitation of Purified IgD by ELISA

Ninety six-well plates (Becton Dickinson) were coated with polyclonal goat anti-mouse IgD (1µg/well) in PBS overnight at 4°C. The wells were washed three to five times in PBS and blocked for 1 hour in PBS containing 1% BSA at 37°C followed by a washing with PBS + 0.05% Tween-20. The wells were then sensitized with 2-fold diluted, purified IgD for 2 hours at 37°C, and were washed extensively in PBS + 0.05% Tween-20. Following washing, plates were incubated with rat anti-mouse IgD (1 µg/well) for 1 hour in PBS at 37°C, washed, and then incubated with HRP-conjugated

goat anti-rat Ig at 75 µl/well for 2 hours at 37°C. Then wells were washed again three to five times in PBS + 0.05% Tween-20, incubated in citrate buffer (pH 4.2, Sigma) containing HRP substrate (ABTS, Sigma) together with 30% H_2O_2 until color developed. A BioRad ELISA reader (BioRad Labs, Life Science Group, Richmond, CA) was used to measure absorbance at 405 nm.

IgD-Receptor Induction

IgD-receptors (IgD-R) were induced *in vivo* by intravenous injections with oligomeric IgD (50-100 µg in saline) 0-3 days prior to the experiments unless otherwise indicated. In some experiments, when IgD was injected together with antigen, it was administered on day-3. Where indicated, IgD-R were induced *in vitro* be exposing T cells to IgD-coated dishes as described previously (Coico et al.,1985). Briefly, the dishes were coated with 0.1-2 µg of oligomeric IgD overnight at 4°C. Incubation of 2.5 x 10^6 splenic T cells in IgD-coated dishes for 18 hr at 37°C led to an increase in the number of T cells expressing receptors specific for IgD.

Immunizations to Analyze B cell Antibody Responses

Where indicated, mice were immunized i.v. with 1 x 10^8 SRBC in 0.1ml of PBS alone or together with oligomeric IgD (50-100 µg) 3 days prior to the day of the experiment. Alternatively, mice were immunized i.v. with 100 µg TNP-ovalbumin (TNP-OVA) alone or together with oligomeric IgD 3 days prior to the day of the experiment.

When goat Ig was used as antigen to prepare goat Ig-primed T cells, animals were primed subcutaneously with 100 µg of goat gamma globulin every 10-15 days, over

a 40 day period. Initial immunizations with goat Ig were performed by emulsifying the antigen in complete Freund's adjuvant (CFA) (Sigma, St.Louis, MO) as described (Cooper et al. 1995). Two ml CFA was mixed with 2 ml of 1 mg/ml purified protein antigen in PBS at 4°C, then CFA/antigen mixture was drawn up into a 3 ml glass syringe with a 19-G needle. The needle was removed and attached syringe to the double-ended locking hub connector (Becton Dickinson, Lincoln Park, NJ) with a 3 ml empty glass syringe at the other end and forced the mixture back and forth from one syringe to the other repeatedly until it formed an emulsion. Then the adjuvant/antigen emulsion was injected into multiple intramuscular (i.m), intradermal (i.d), or subcutaneous (s.c) sites of the mouse. Subsequent injections were given in incomplete Freund's adjuvant (IFA) (Becton Dickinson, Lincoln Park, NJ). The final injection was given i.v. in the absence of adjuvant, 3 days prior to the day of experiment. Where indicated, IgD (50-100 μg) together with antigen was administered to these goat Ig-primed mice on day -3 relative to the day of the experiment.

Fractionation of Spleen Cells

Splenic T cells were purified by immunomagnetic separation using goat anti-mouse Ig-coated magnetic beads purchased from Miltenyi Biotec, Inc. (Auburn,CA). Three ml of PBS+2% FBS plus 1ml of $3x10^7$ spleen cells were placed on non-coated dishes and incubated at 4°C for 1.5 h, then the non-adherent cells were harvested and adjusted the cell concentration to $1x10^7$/ml. Cells were washed twice with PBS+5mM EDTA+0.5% bovine serum albumin, resuspended in 80 μl PBS+5 mM EDTA +5% BSA (MACS separation buffer) and were labeled with 20μl MACS goat-anti-mouse IgG

microbeads and incubated for 45 min at 4°C. The MinMACS magnetic separation column was washed once with 500µl separation buffer, 100 µl magnetically labeled cell suspension was applied to the column and allowed the suspension to pass through and the effluent cells were collected. Approximately 90% of effluent cells were T cells as assessed by FACS analysis ($CD3^+$, $B220^-$). The cell concentration was adjusted to 1 x 10^6/ml for proliferation assays. Alternatively, splenic T cells were isolated by goat anti-mouse IgG + IgM-coated 100 mm Petri dishes (Fisher Scientific Co. Pittsburgh, PA). Briefly, dishes were coated with 50 µg of goat anti-mouse IgG + IgM in 4 ml 0.05 M Tris Buffer, pH 9.5, at room temperature for 1h or overnight at 4°C, washed the coated dishes 5 times with PBS and one time with PBS+2% FBS just before use. One ml of 3 x 10^7 spleen cells plus 3 ml of PBS + 2% FBS were added into non-coated tissue culture grade Petri dishes, incubated at 4°C for 1.5 h, then non-adherent cells were transferred into goat anti-mouse IgG+IgM-coated dishes, and incubated at room temperature for 30 minutes. After incubation, non-adherent cells (isolated T cells) were harvested and washed twice with PBS + 2% FBS and adjusted to 1 x 10^6/ml for the proliferation assay. Approximately 80% of non-adherent cells were found to be $CD3^+$, $B220^-$ as assessed by FACS analysis. The fractionation procedure was repeated once to achieve a 92-98% $CD3^+$ T cell population.

Splenic B cells were also prepared by immunomagnetic separation using microbeads coupled to anti-Thy 1.2 Mab. One ml of 3 x 10^7 spleen cells plus 3 ml of PBS + 2% FBS were added into non-coated dishes, incubated at 4°C for 1.5 h, harvested the non-adherent cells, and adjusted the cell concentration to 1x10^7/ml. Cells were

washed twice with PBS + 5 mM EDTA + 0.5% FBS, respended the cell pellet in 90 μl PBS + 5 mM EDTA + 5% FBS (MACS separation buffer), labeled the cells with 10 μl MACS rat anti-mouse Thy1.2 microbeads, and incubate for 45 minutes at 4°C. MinMACS magnetic separation column was washed once with 500 μl separation buffer, 100 μl magnetically labeled cell suspension was applied to the column and allowed the suspension to pass through and collected the effluent cells. Approximately 90-95% of effluent cells were B cells ($B220^+$, $CD3^-$) as assessed by FACS analysis. Cell concentration was adjusted to 1 x 10^6/ml for proliferation assays. We also used complement-mediated cytolytic elimination of T cells and isolated splenic B cells as follows: 1 ml of 1 x 10^7 spleen cells were incubated with anti-mouseThy1.2 (Cedarlane, Hornby, Ontario) (final dilution to 1:20) on ice for 1 h; cells were pelleted at 4°C, 1200 rpm for 10 minutes, then 1 ml of rabbit complement (Cedarlane, Hornby, Ontario) at 1:10 dilution was added and the suspension was incubated at 37°C for 1 h. Isolated B cells were washed twice with PBS + 2 %FBS and adjusted the concentration to 1x10^6/ml for the proliferation assay. Approximately 95% of purified B cells were $B220^+$, $CD3^-$ as assessed by FACS analysis.

Isolation of Peritoneal Macrophages

Peritoneal adherent cells were prepared by adherence to plastic according to Fortier et al (1995). One ml of 3% Brewer's thioglycollate medium (Sigma, St. Louis, MO) was injected into the peritoneums of the mouse 5 - 7 days prior to cell harvest. Mice were sacrificed and then 10 ml RPMI1640 medium was injected i.p. to lavage

peritoneal fluid. Peritoneal cells were washed twice with RPMI1640 medium 10 min at 1200 rpm, 4°C. The cell pellet was resuspended in RPMI1640 medium containing 10 % FBS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 pg/ml recombinant IL-1β (complete RPMI). Then 2 x 10^5cells/well were plated into 96-well plate for 2 h at 37°C, non-adherent cells were removed by tirturation. Finally, the peritoneal adherent cells were pulsed with antigen for the proliferation assays.

Plaque-Forming Cell (PFC) Assay

Anti-SRBC antibody-forming cells derived from in vitro cultures were assayed by the slide modification technique of Jerne et al (1978). Initially, microscope slides were coated with a thin film of boiled 0.1% agarose. Slides were allowed to air dry. Five ml of SRBC was washed with 45 ml HBSS by centrifuging for 10 minutes at 3000 rpm at least three times until the supernatant was clear. One ml SRBC pellet was resuspended in 9 ml HBSS to a final concentration 10% for PFC assay. Then 0.5% pre-warmed agarose (42°C) was dispensed as 0.5 ml/tube to each 5 ml tube, and the following were also added: 100 μl 10% SRBC, 100 μl of 2 x 10^6 spleen cells. Tubes were votexed and the mixture was poured onto slides, slides were allowed to dry, and were placed into slide-holding rack and incubated at 37°C warm room for 1.5h. After incubation, fresh, diluted (1:20 dilution in HBSS), SRBC-absorbed guinea pig complement (GIBCO-BRL, Grand island, NY) was added to the slides in the racks and incubated rack in 37°C warm room for 1.5 h. Finally, slides were removed from the

incubator and plaques were counted using a dissecting microscope at 10x magnification. PFCs were calculated using the following formula:

PFC/Spleen = PFC/slide x (total number of spleen cells/spleen cells per slide)

For example: if PFC/slide = 50, total number of spleen cell = $3x10^7$, spleen cells per slide=$2x10^5$, then PFC/Spleen = 50 x ($3x10^7$/ $2x10^5$) = 50 x 150 = 7500

Preparation of SRBC-Absorbed Guinea Pig Complement

Ten ml of 10X lyophilized guinea pig complement was dissolved in 10 ml ice cold ddH_2O. One part SRBC and 2 part complement was mixed, incubated on ice for 1 h, and then centrifuged for 10 minutes at 3000 rpm, 4°C. Absorbed supernatants were frozen as 5 ml aliquots at -70°C.

ELISPOT Assays

Spleen cells from SRBC or TNP-OVA-immunized mice were assayed for anti-SRBC or anti-TNP-OVA antibody responses using a modified ELISPOT assay (Coico and Licke, 1996). Briefly, nitrocellulose (NC) membranes were soaked in PBS containing 7.5%, 10%, or 15% SRBC lysates prepared as follows: After washing 5 ml SRBC in 45 ml PBS, 2 ml of SRBC pellets were prepared. Cells were lysed by adding 5 ml sterile water to the 2 ml SRBC pellet. Lysed SRBC were pelleted again and resuspended in 8 ml 1x PBS to yield a 20% suspension. Lysates were centrifuged at 4°C, 14,000 rpm for 30 min, diluted with PBS and then used to coat NC membranes overnight at 4°C. NC membranes were then washed three times in PBS and soaked for 1hour (blocking step) with in PBS containing 1% BSA. Prior to placing SRBC lysate-coated

NC membranes into the blotting minifold apparatus (Schleicher & Scheull), they were washed three times in PBS. Cells from SRBC-immunized or control mice were added at densities of 10^5 or 10^4 cells/well in PBS in five well replicates and incubated three hours at 37°C. NC membranes were then removed, washed extensively in PBS to remove cells, then incubated in HRP-conjugated GAM Ig for 1 hour. NC membranes were washed three times in PBS, then incubated in 50 mM sodium acetate buffer (pH 5.0) containing HRP substrate (AEC, Sigma) together with 30% H_2O_2 until spots developed. NC membranes were also coated with 1% BSA and used in parallel with the SRBC-coated membranes as controls. Anti-SRBC antibody forming cells (ELISPOTS) were enumerated microscopically and the results expressed as the number of anti-SRBC ELISPOTS/spleen. In some experiments, NC membranes were coated with 10 μg/ml, 25 μg/ml, or 75 μg/ml TNP-OVA instead of SRBC lysates to measure anti-TNP-OVA ELISPOTS/spleen.

Cell cultures

Responses of T cells from goat Ig-primed BALB/C, $IgD^{-/-}$, and $IgD^{+/-}$ mice were measured in antigen-presenting cell (APC) assays using goat Ig-pulsed B cells as follows: Splenic T cells from goat Ig-primed control mice or mice treated one day earlier with oligomeric IgD (50-100 μg) were adjusted to1 x 10^6/ml in RPMI medium containing 10% FBS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 pg/ml recombinant IL-1β (complete RPMI). Splenic B cells were prepared in complete RPMI medium, irradiated with 2000R, aliquoted into wells (1 x 10^5/well) with or without GAM Ig, GAM IgM, or GAM IgD (10 μg/ml), and

preincubated for 1 h at 37°C. T cells were added to the wells (1 x 10^5/well) and cultures were incubated for 72 h then labeled with 1μCi ^{3}H-TdR for additional 6 hours, harvested, and analyzed in a scintillation counter.

In some experiments, unfractionated SRBC-primed spleen cells (2 x 10^5/well) from control mice or mice treated with oligomeric IgD were cultured for 72 hours and labeled with 1μCi ^{3}H-TdR for additional 6 hours in complete RPMI medium with or without SRBC (20 μl of a 0.1% SRBC suspension), harvested, and analyzed in a scintillation counter.

Statistical Analysis

Significance of difference between groups was determined by student's t-test.

Flow Cytometry

IgD-R expression was detected by treating purified splenic T cells with biotinylated-IgD (0.5 μg/1 x 10^6 cells) for 30 minutes on ice. The cells were then washed three times with PBS containing 1%BSA and 0.01% sodium azide (staining buffer) then treated for 30 minutes with avidin-FITC diluted 1:100 in staining buffer. After washing three times with staining buffer, cells were analyzed using a Coulter Elite Cell Sorter (Coulter Corp., Hialeah, FL). T cells treated with avidin-FITC alone were used in parallel with biotinylated-IgD/avidin-FITC staining as controls.

The purity of isolated splenic T cells was analyzed by staining cells separately with anti-CD3-FITC and anti-B220-FITC. Isolated T cells were treated with anti-CD3-

FITC, B222-PE (0.5 μg/1 x 10^6cells) or isotype control for 30 minutes on ice. The cells were then washed three times with staining buffer and were analyzed by FACS.

The purity of isolated splenic B cells was analyzed by staining with B220 -FITC and anti-CD3-FITC. Isolated T cells were treated, in separate staining tubes, with B220-FITC and anti-CD3-FITC (0.5 μg/1 x 10^6cells) or isotype control for 30 minutes on ice. The cells were then washed three times with staining buffer and were analyzed by FACS.

The effects of IgD-R upregulation on CD28 and CD40L expression was measured by examining cell surface expression of these determinants by dual fluorescence staining. Briefly, the purified splenic T cells (2 x 10^7 cells) were treated in vitro with in RPMI medium supplemented with: 1) nothing (negative control); 2) oligomeric IgD (10 μg/ml); or, 3) anti-CD3 (0.5μg/ml). Cells were incubated at 37°C, 5% CO_2 for 4 hours or 12 hours. In some experiments, splenic T cells isolated from BALB/c mice that were treated one day earlier with oligomeric IgD in vivo were used to measure the possible effects of IgD-R upregulation on CD28 and CD40L expression. CD28 and CD40L expression was detected by staining T cells with hamster anti-mouse CD28-PE/rat anti-mouse CD4-FITC, hamster anti-mouse CD40L-PE/rat anti-mouse CD4-FITC as follows: T cells (1 x 10^6 cells) were incubated with rat anti-mouse CD4-FITC (1 μg/1 x 10^6 cells) for 30 minutes on ice. 1 x 10^6 cells were then washed three times with staining buffer (PBS + 1% BSA + 0.01% NaN_3), and treated with 1 μg of hamster anti-mouse CD28-PE or hamster anti-mouse CD40L-PE for 30 minutes on ice. After washing three times with staining buffer, cells were analyzed by FACS. T cells treated with rat Ig-FITC or hamster Ig-PE alone were used as isotype controls.

The expression of Fas and Fas ligand was analyzed by staining with anti-Fas-FITC and anti-Fas-ligand-PE. Cells were labeled with anti-Fas-FITC (1 μg/1 x 10^6 cells) following overnight stimulation for 30 minutes on ice. The cells were then washed three times with staining buffer, and incubated with anti-Fas-ligand-PE (1 μg/1 x 10^6 cells) for 30 minutes on ice. After washing three times with staining buffer, cells were analyzed by FACS.

Intracellular cytokines were analyzed by staining. Briefly, purified splenic T cells (1 x 10^6cells) were treated with rat anti-mouse CD4-PE, rat anti-mouse CD4-FITC, or isotype control rat Ig-FITC or rat Ig-PE (1 μg/1 x 10^6 cells) for 20 minutes at 4°C, and were then incubated with 100μl of fixation reagent A for 20 minutes at room temperature in dark. The cells were then washed once in 3 ml PBS + 0.1% NaN_3 + 5% FBS, and were treated with 100 μl permeabilization reagent and FITC- or PE-conjugated rat anti-mouse INFγ, rat anti-mouse IL-2, rat anti-mouse IL-4 and rat anti-mouse IL-5 (1 μg/1 x 10^6 cells) or FITC and PE-conjugated rat Ig isotype controls for 20 min at room temperature in the dark. Cells were washed once with 3 ml PBS + 0.1% NaN_3 + 5% FBS, and were reconstituted with 1ml PBS + 0.1% NaN_3 + 5% FBS and analyzed by FACS. The staining was measured on a logarithmic scale. A minimum of 1 x 10^4 events were collected per sample.

RNA Extraction

Before RNA extraction, 10 x 10^6 cells were treated with: 1) medium; 2) PMA (10 ng/10^6 cells) + ionomycin (400 ng/10^6 cells); 3) oligomeric IgD (10 μg/ml); and, 4)

anti-CD3 Mab (1 μg/ml). Cells were incubated at 37°C for 4 h. Following stimulation, cells were washed twice with PBS. 5-10x10^6 cells were then lysed in 1 ml TRI REAGENT (Sigma) by repeated pipetting. After homogenization, the insoluble material (extracellular membranes, polysaccharides, and high molecular weight DNA) was removed by centrifugation at 12,000 rpm for 20 minutes at 4°C and the supernatant containing RNA and protein was transferred to a fresh tube. The samples were allowed to stand for 5 minutes at room temperature, then 0.2 ml of chloroform (Sigma) per ml of TRI REAGENT was added and was then shaken vigorously for 15 seconds. The samples were allowed to stand for 2-15 minutes at room temperature then centrifuged at 12,000 rpm for 30 minutes at 4°C to separate the mixture into 3 phases: a red organic phase containing protein; an interphase containing DNA; and, a colorless upper aqueous phase containing RNA. The RNA-containing aqueous phases were transferred to a fresh DEPC (Sigma)-treated tube and 0.5 ml of isopropanel (Sigma) per ml of TRI REAGENT was added to the samples. They were then mixed well and incubated for 15 minutes at room temperature. Tubes with RNA were centrifuged at 12,000 rpm for 30 minutes at 4°C, and supernatants were discarded. RNA pellets were rinsed with 75% ethanol, and centrifuged at 12,000 rpm for 15 minutes at 4°C. RNA pellets were allowed to dry for 5 minutes, resuspended RNA in DEPC-treated water or saved the RNA pellet in 75% ethanol at -80°C. RNA concentration was measured by spectrophotometer (O.D.at 260 and 280 nm) using the following formula:

Concentration= $O.D._{260}$ x 40 x dilution factor

RNA Protection Assay (RPA)

A commercial mouse cytokine mCK-1 multi-probe template sets (in vitro transcription kit for transcription reaction) was used to measure cytokine profiles of T cell populations under study. In addition to cytokine-specific probes (see below), the RPA kits included: proteinase K, hybridization buffer, RNase for RPA analysis, mL32 housekeeping template, and mGAPDH housekeeping templates. RPA kits were purchased from Pharmingen (San Diego, CA).

Preparation of cytokine probes: 4 μl 5x transcription buffer 1 μl 200 mM DTT+ 2 μl 3NTP mix (ATP, UTP, GTP) + 10 μl [α-^{32}P] CTP (10 mCi/ml, 400-800 Ci/ml) + 1 μl placental ribonuclease inhibitor (20-40 U) + 1 μl 0.5 mg/ml mck-1 multi-probe template DNA + 1 μl T7 RNA polymerase were added to an autoclaved centrifuge vial, and incubated at 37°C for 30-60 minutes. After reaction, the vial was incubated with 10 U RNase-free DNase I at 37°C for 15 minutes to remove template DNA. Then 2 μl of 10 mg/ml tRNA and water were added to a final volume of 50 μl. Following extraction with phenol / chloroform / isoamyl alcohol, aqueous phase was transferred to a clean microcentrifuge tube containing 1 μl of 10 mg/ml yeast tRNA and incubated with 200 μl of 2.5 M ammonium acetate plus 750 μl of 100% ethanol on ice for 15 minutes. After incubation, RNA was precipitated by centrifugation at 14,000rpm for 15 minutes at 4°C. The pellet was washed once with 50 μl water plus 200 μl of 2.5 M ammonium acetate and 750 μl of 100% ethanol and finally rinsed with 75% ethanol/25% 0.1 M sodium acetate, pH 5.2. The pellet was dissolved in 100 μl hybridization buffer and used 1 μl for

counting in liquid scintillation counter to determine incorporation for the next step hybridization.

Hybridization of RNA probe to sample RNAs: Sample RNA pellets were dissolved in 30 µl hybridization buffer containing 5 x 10^5 cpm of RNA probe, incubated for 5 minutes at 85°C to denature RNA and then immediately transfered to desired hybridization temperature at 45°C for overnight (>8hr). After overnight hybridization, the sample was incubated with 350 µl ribonuclease digestion buffer containing 40 µg/ml ribonuclease A and 2 µg/ml ribonuclease T for 30-60 minutes at 30°C to digest single strand RNAs. The sample was then incubated with 10 µl of 20% (wt/vol) SDS and 2.5 µl of 20 mg/ml proteinase K for additional 15 minutes at 37°C, then extracted once with 400 µl phenol / chloroform / isoamyl alcohol, and transferred the aqueous phase to a clean microcentrifuge tube containing 1 µl of 10 mg/ml yeast tRNA. Hybridized RNAs were precipitated by adding 1ml of 100% ethanol. Finally, hybridized RNAs were dissolved in 3-5 µl RNA loading buffer and analyzed them on a denaturing polyacrylamide/urea sequencing gel.

Probes contained in the RPA kit measured the following cytokines:

IL-4, IL-5, IL-10, IL-13, IL-15, IL-9, IL-2, IL-6, INF-γ

Biotin-Labeling of 7C5-E1 T Hybridoma Cells.

T hybridoma cells (1 x 10^8) were washed three times with PBS-Ca-Mg buffer. Cells were treated with 6 ml of biotinylation solution (15 µl freshly prepared sulfo-NHS-biotin to 6 ml of ice cold PBS-Ca-Mg buffer [PBS containing 1 mM $MgCl_2$ and 0.1mM $CaCl_2$] to final concentration of 0.5 mg/ml) at 4°C for 30 min. Samples were centrifuged

at 1200 rpm for 10 min at 4°C to remove extra biotin, then cells were treated with cold serum free RPMI1640 medium to quench residual free sulfo-NHS-biotin on ice for 5-10 min. The cells were washed three times with cold PBS-Ca-Mg buffer. Finally, cells were lysed in 0.5 ml lysis buffer (50 mM Tris-Cl, pH7.4, 1% NP40, 0.25% sodium dexycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 10 μg/ml aprotinin (Sigma), 10 μg/ml leupeptin (Sigma), 1 mM $NaVO_3$, 1 mM NaF, 2 mM EDTA, 10% glycerol), vortexed and incubated on ice for 20 min. Lysates were vortexed again and microfuged at 14000 rpm for 20 min at 4°C. Supernatants were transferred to fresh tubes and either processed immediately or stored at -80°C.

Preparation of 7C5-E1 Cell Lysates

Before the lysate preparation, 20-40 x 10^6 7C5 cells were treated with: 1) medium; 2) PMA (100 ng/10^6 cells) + ionomycin (1 μm); 3) anti-CD3 Mab (1 μg/10^6 cells); and, 4) oligomeric IgD (10 μg/10^6 cells). The cells treated with anti-CD3 and oligomeric IgD were preincubated on a rotator at 4°C for 45 minutes, then cells were transferred to 37°C water bath and incubated for 5 minutes. Following stimulation, cells were quickly placed on ice, and washed 3-4 times in ice cold PBS + 1 mM sodium orthovanadate. Cells were then lysed in ~ 25 μl or 500 μl lysis buffer to prepare for immunopricipitation (see below), vortexed and incubated on ice for 20 minutes. Lysates were voxtexed again and microfuged at 14000 rpm for 20 minutes at 4°C. Supernatants were transferred to fresh tubes and either processed immediately or stored at -80°C.

Coupling IgD and IgG to Sepharose-4B

CNBr-activated Sepharose-4B (Sigma) was incubated in 1 mM HCl for15 minutes at room temperature and allowed to swell. Resin was washed with 1 mM HCl, 200 ml per 1g dry gel and coupling buffer (0.1 M bicarbonate, pH8.3, containing 0.5 M NaCl), 5 ml per 1g dry gel on scintered glass filter. Following washing, Sepharose-4B was immediately mixed with protein solution and incubated overnight at 4°C. Following overnight incubation, gel was washed with coupling buffer (30X gel volume) then incubated in coupling buffer containing 0.2 M glycine at room temperature for 2 hours to block unreacted sites. Gel was washed with coupling buffer again (30X gel volume) and then washed with 0.1 M acetate buffer pH 4.0 containing 0.5 M NaCl (30X gel volume). Finally, gel was washed again with coupling buffer and stored the gel in storage buffer (PBS + 0.01% NaN_3) at 4°C.

Immunoprecipitation of Cell Lysates

500 µl of 7C5 cell lysates were precleared with 80 µl protein A+G agarose (Sigma) at 4°C for 4 hours, then precleared with 80 µl mIgG-Sepharose (4 mg/ml) at 4°C for 4 hours followed by immunoprecipitated with 100µl mIgD-sepharose (3.5 mg/ml) overnight at 4°C. After immunopercipitation, beads were washed extensively with lysis buffer and PBS, then added the 3X -reduced or nonreduced sample buffers heating 5 minutes at 100°C, microcentrifuged for 5 minutes and the supernatants were analyzed by SDS-PAGE under reducing conditions. Following immunoblotting (Western blots, see below), blots were probed with biotinylated-mIgD (1:2000 dilution), followed by HRP-streptoavidin (1:1000 dilution). If cell lysates were immunoprecipitated with anti-phosphotyrosine antibody (Upstate Biotechonology, Lake Placid, NY) coupled to protein

A+G agarose, then the Western blots were probed with anti-phosphotyrosine antibody (1:2000 dilution), followed by HRP-conjugated secondary antibody (1:2000 dilution). Blots were washed extensively between incubation and developed using the ECL system.

SDS-PAGE and Western Blots

The molecular weight of IgD-R was estimated by SDS-PAGE. Briefly, samples were subjected to electrophoresis under reducing conditions or non-reducing conditions at 180V for ~ 1 hour. When electrophoresis was completed, the gels were equilibrated in transfer buffer for 30 minutes, followed by assembly of the transfer sandwich: in layers of wet filter paper, gel, PVDF membrane, and filter paper in transfer buffer, proteins were transferred electrophoretically from gel to the membrane at 25V in a transfer tank filled with cold transfer buffer at room temperature overnight. After transfers, the membranes were washed three times with PBS and blocked with blocking buffer (PBS + 5% dry milk) for 1 hour at room temperature. Then the membranes were incubated with biotinylated-IgD (1:2000 diluted in freshly prepared blocking buffer), or with anti-phosphotyrosine antibody (1:2000 diluted in freshly prepared blocking buffer) at room temperature for 2 hours. Membranes were washed eight to ten times with PBS + 0.5% Tween-20 and two times with PBS before incubation with HRP-streptoavidin (Pharmigen, San Diego, CA) or HRP-conjugated secondary antibody for 2 hours at room temperature. Finally, membranes were washed extensively and developed using an ECL western blots detection system (Upstate Biotechnology, Lake Placid, NY).

Apoptosis

In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer surface of the plasma membrane, thereby exposing PS to the external cellular environment (Vermes et. al. 1995). Annexin V is a 35~ 36 kDa Ca^{2+} dependent phospholipid- binding protein that has a high affinity for PS and binds to cells with exposed PS. Thus fluorochrome-conjugated Annexin (e.g. Annexin- FITC) serves as sensitive probe for flow cytometric analysis of cells that are in the early stages of apoptosis. The percentage of apoptotic cells was always greater than the percentage of typan blue positive cells and the appearance of significant numbers of apoptotic cells preceded the decline of cellular viability. The detection of apoptotic cells by Annexin V is technically simple and can be performed on fixed cells. One major concern with this assay is the lack of a control molecule to assess the amount of non-specific binding and it has been recommended in the kit to utilize unconjugated Annexin to block the binding of labeled Annexin V-FITC. Propidium Iodide staining solution is used to assess plasma membrane (PM) integrity in Annexin V apoptosis assay. PI does not cross the PM of the cells that are viable or in the early stages of apoptosis (Nicoletti et. al. 1991; Zamai et. al. 1996). PI is detected in the orange range of the spectrum using 562-588 nm band pass filter.

Cells were washed twice with cold PBS and respended in 1 x binding buffer (10X binding buffer: 0.1 M HEPES/NaOH. pH 7.4; 1.4 mM NaCl; 25 mM $CaCl_2$, diluted to 1X prior to used) at a comcentration of 1 x 10^6 cells/ml and stained according to manufacturer's instructions. 100 µl of the solution was transferred to a 5 ml culture tube; Annexin V- FITC, or PI and Annexin V-FITC/PI were added, gently mixed the cells, and

incubated for 15 minutes at room temperature in the dark. Then 400 µl of 1X binding buffer was added to each tube. Samples were analyzed by flow cytometry. Unstained cells and cells treated first with unconjugated Annexin- V followed by Annexin V- FITC staining were used as controls.

Recipes for Buffers and Solutions

DEPC-treated water:

1 ml DEPC was mixed with 1 liter dH_2O, stirred overnight in the hood and then autoclaved.

Phosphate-buffered saline (PBS):

0.23 g NaH_2PO_4 (anhydrous) (1.9 mM), 1.15 g Na_2HPO_4 (anhydrous) (8.1 mM), 9.00 g and NaCl (154 mM) were dissolved in 900 ml dH_2O, pH was adjusted to 7.0 using 1 M NaOH or 1 M HCl, brought up to 1 liter volume, and then filter sterilized.

1 M $CaCl_2$:

11.09 g $CaCl_2$ was dissolved in 40 ml PBS, and brought up to 100ml total volume with PBS. Filter sterilized and stored at room tempreture.

1 M D-galactose ($C_6H_{12}O_6$ Mr.180.2):

18.02 g D-galatose was dissolved in 40 ml PBS, and brought up to 100ml total volume with PBS. Filter sterilized and stored at 4°C.

PBS containing 1 mM CaCL2, pH 7.0 (PBS-CA buffer):

900 ml PBS and 1ml 1M CaCL2 were mixed, adjusted to pH7.0 using 1M NaOH or 1M HCl. PBS was added to make up to 1 liter. Filter sterilized and stored at 4°C.

PBS-CA containing 0.1M D-galactose, pH7.0 (PBS-CA-GAL):

900 ml PBS-CA buffer and 100 ml 1M D-galactose were mixed, adjusted to pH 7.0 using 1 M NaOH or 1 M HCl. PBS-CA buffer was added to bring up to 1 liter. Filter sterilized and stored at 4°C.

0.28 M Cacodylic buffer:

2.24 g cacodylate was dissolved in 30 ml dH_2O, pH was adjusted to 7.4 using 1 M NaOH or 1 M HCl. dH_2O was added to make up to 50 ml. Filter sterilized and stored at 4°C.

Preparation of TNP-OVA:

Three ml of OVA (Sigma) was prepared at 100 mg/ml in PBS. It was dialyzed overnight against 1 liter PBS. 80 mg 2,4,6 TNP-S (picrylsulfonic acid) was added to 15 ml of the cacodylic buffer. Three ml of dialyzed OVA was mixed with 15 ml 2,4,6 TNP-S (final concentration of OVA was 300 mg/18ml =16.66 mg/ml) and incubated in dark for 3h. Dialyzed against 2 liter saline (4 times changes). TNP-OVA was stored in 2 ml aliquots at -20°.

5x SDS/electrophoresis buffer (5x Running buffer):

15.1 g Tris base, 72.0 g glycine and 5.0 g SDS were dissolved in 1000 ml dH_2O. It was stored at 4°C until use.

4x Tris.Cl/SDS, pH 6.8(0.5 M Tris.Cl containing 0.4% SDS):

6.05 g Tris base was dissolved in 40 ml dH_2O; pH was adjusted to 6.8 with 1N HCl. Volumes were brought up to 100 ml by adding dH_2O, filter-sterilized, supplemented with 0.4 g SDS, and stored at 4°C.

4x Tris.Cl/SDS, pH 8.8 (1.5 M Tris.Cl containing 0.4% SDS):

91 g Tris base was dissolved in 300 ml dH_2O, pH was adjusted to 8.8 with 1N HCl. The solution was brought up to 500 ml by adding dH_2O, filter-sterilized, supplemented with 2 g SDS, and stored at 4°C.

2x SDS/sample buffer:

25 ml 4x Tris.Cl/SDS, pH 6.8, 20 ml glycerol, 4 g SDS, 2ml 2-ME or 3.1 g DTT, 1 mg bromphenol blue were mixed, and dH_2O was added make up to 100 ml. The buffer was filter- sterilized and stored in 1ml aliquots at -70°C.

6x SDS/sample buffer:

7 ml of 4x Tris.Cl/SDS, pH 6.8, 3.8 g glycerol (~3ml), 1 g SDS, 0.93 g DTT, 1.2 mg bromphenol blue were mixed, dH_2O was added to make up to 10 ml. The buffer was filter-sterilized and stored in 0.5ml aliquots at -70°C.

RESULTS

Part I: The roles of IgD-receptor and B cell IgD in antigen presentation.

In vivo and *in vitro* studies of immunoregulatory properties of IgD were made possible by the use of murine IgD-secreting plasmacytomas. The plasmacytoma TEPC1017, which secretes oligomeric IgD, was maintained by intraperitoneal transfer in pristane-primed BALB/c mice. IgD was routinely purified from IgD-containing peritoneal ascites by affinity chromotography using GS-1 Sepharose column and their purity and integrity were checked by ELISA and SDS-PAGE. In mice, IgD-R is present on a small percentage (<5%) of T cells in spleens and lymph nodes. Injection of oligomeric IgD results in rapid increases in the number of $CD4^+$ IgD-R^+ T cells (~30-40%) and the affinity of IgD-R also increased as measured by immunofluorescence using biotinylated-IgD and avidin-FITC. Moreover, direct plaque assays and ELISPOT assays demonstrated that treatment with oligomeric IgD enhanced primary and secondary antibody responses to antigens such as SRBC and TNP-OVA, in normal but not athymic mice. The identification of IgD-R expressed on T cells following their exposure to oligomeric IgD has suggested a role for these receptors in T-B interactions that might explain the immunoregulatory properties IgD-R^+ T cells. We studied the mechanism by which IgD-R^+ T cells facilitated antibody responses by examining whether T cells also benefit from their expression of IgD-R. Experiments were designed to determine whether upregulation of IgD-R on T cells facilitates antigen presentation by IgD^+ B cells. Goat Ig-primed splenic T cells from BALB/c mice were tested for their ability to respond to antigen-presenting B cells treated with goat anti-mouse (GAM) IgM or GAMIgD. T cells response to GAMIgM and GAMIgD

presented by B cells were significantly higher when goat Ig-primed cells were induced to express IgD-R by exposure to oligomeric IgD compared with goat Ig-primed control T cells. This effect was inhibited when monomeric IgD was added to the cultures. No differences in T and IgD-R^+ T cells responses were seen using adherent cells as APCs. B cells from $IgD^{-/-}$ mice were also tested. Such B cells present antigen to IgD-R^+ T cells without promoting enhanced responses compared with B cells from heterozygous $IgD^{+/-}$ mice. These studies suggest that IgD may play a costimulatory role during antigen presentation. We conclude that when T cells are induced to express IgD-R, these lectin like receptors can ligate B cells membrane IgD during antigen presentation to facilitate responses of each of the cells engaged in cognate interaction, yielding enhanced antigen-specific T cell and B cell responses.

1. *Quantitation of purified TEPC1017 IgD by ELISA, SDS-PAGE.*

IgD was routinely purified on GS-1-Sepharose columns and its purity and concentration IgD assessed by ELISA (figure 1A). SDS-PAGE was also used to check the purity and integrity of IgD (figure 1B).

2. *Competitive inhibition of IgD-R staining with IgD.*

In previous studies, we have relied on a rosetting technique to quantitate T cells expressing IgD-R (Coico et. al .1985). In this study, expression of IgD-R was measured by flow cytometry (immunofluorescence) using biotinylated-IgD and avidin-FITC. As shown in figure 2, T cells isolated from BALB/c mice that were treated one day earlier with oligomeric IgD *in vivo*, and stained with biotinylated-IgD / avidin-FITC. Analysis of T cells subsets revealed that these IgD-R^+ cells were $CD4^+$, $CD8^-$, consistent with previously reported results using rosetting assays (Coico et. al. 1985). FACS analysis revealed an increased frequency of IgD-R^+ cells as compared

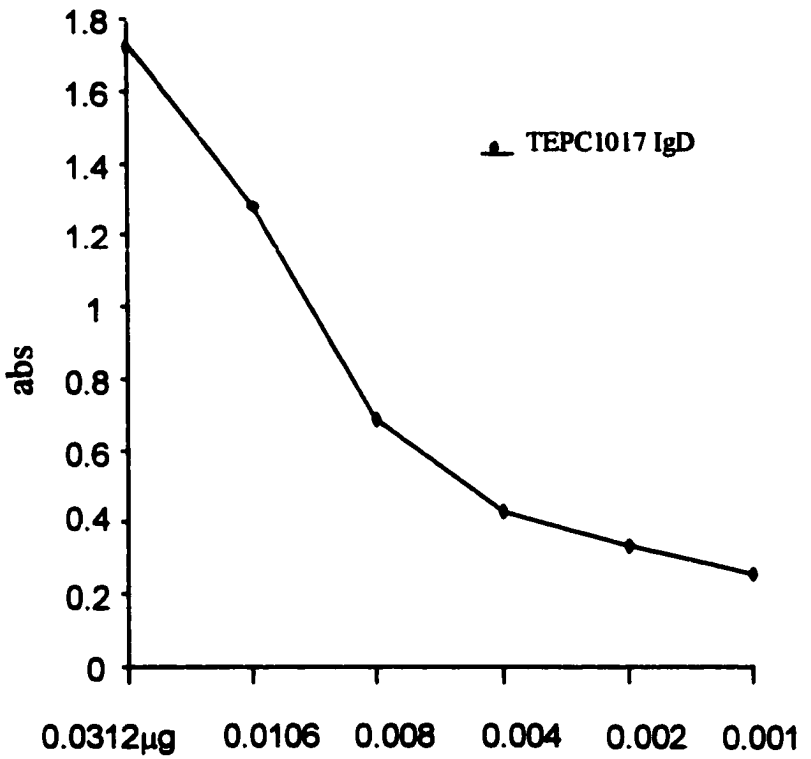

Figure 1A. ***Quantitation of purified TEPC1017 IgD by ELISA.*** Ninety six wells were coated overnight at 4°C with 1μg of polyclonal goat anti-mouse IgD in PBS. Wells were washed in PBS and incubated with PBS containing 1% BSA for 1h as a blocking step. Wells were washed with PBS + 0.5% Tween-20 and incubated with titrated purified IgD. After 2h incubation, wells were washed with PBS containing 0.05% Tween- 20, then 1μg of rat anti-mouse IgD was added and incubated for 1 hr. Wells were then washed and 75μl of HRP-conjugated goat anti-rat Ig was added for 2 hr. Wells were washed again and incubated in citrate buffer, pH 4.2, containing HRP substrate ABTS together with 30% H_2O_2 until color developed. Quantitation of Elisa result was performed using a BioRad ELISA reader at 405nm.

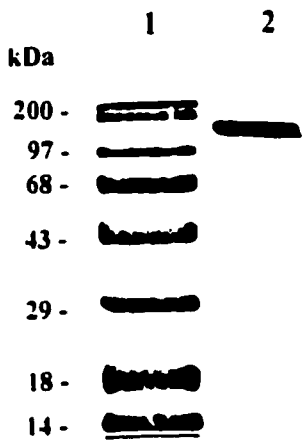

Figure 1B. ***SDS-PAGE analysis of purified TEPC1017 IgD.*** IgD was purified by affinity chromatography by utilizing GS-1-Sepharose column as described in materials and methods. Purified IgD was run on a 10% SDS-PAGE gel under nonreduced conditions (lane 2). Standard m.w. marker was also run (lane 1).

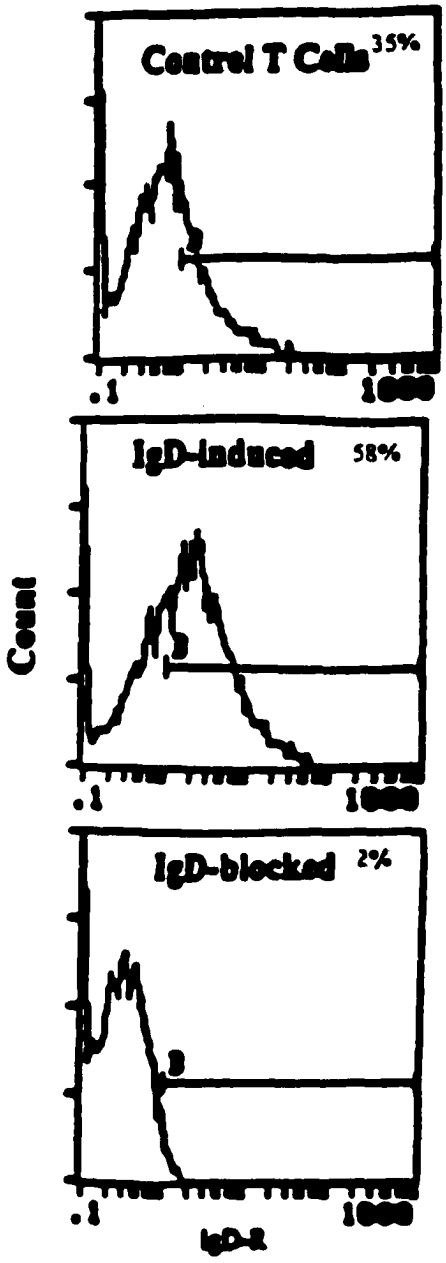

Figure 2. ***Detection and competitive inhibition of IgD-R on T cells.*** T cells were isolated from control BALB/c mice and stained with biotinylated-IgD followed by avidin-FITC (top). Alternatively, T cells were isolated from BALB/c mice injected i.p. with 50 μg of oligomeric IgD on day −1 and then stained for IgD-R in the absence (middle) or presence (bottom) of unconjugated IgD (200 μg). Results shown are representative of five experiments performed.

with control T cells. The results show an increased expression of IgD-R from 35% in control T cells to 58% in IgD induced T cells. Moreover, incubation of T cells with unconjugated IgD (200 μg or 100 μg) prior to staining with biotinylated-IgD completely blocked staining with biotinylated IgD, only 2% of T cells are IgD-R$^+$ and showed that unconjugated IgD block the interaction between membrane IgD-R and biotinylated-IgD (figure 2, lower panel). The results are consistent with competitive inhibition data obtained using Tδ cells rosetted with IgD-coated sheep erythrocytes which do not form rosettes when soluble IgD was used to block binding (Coico et. al . 1985). Other Ig isotypes failed to block this rosetting (Coico et. al. 1990; Tamma and Coico, 1992)

3. *Dose dependent inhibition of IgD-R and IgD interaction.*

In previous studies, we have shown that splenic T cells exhibit an increased frequency of IgD-R expression after exposure to IgD by rosetting assays (Coico et. al. 1985c; Coico et. al. 1988). However, we were unable to determine whether the affinity of IgD-R is also increased following IgD-R crosslinking. In this study, affinity of IgD-R were measured in a dose dependent manner by immunofluorence staining using biotinylated-IgD and avidin-FITC. Details of *in vivo* treatment of mice with oligomeric IgD are as described in general materials and methods section. As shown in figure 3, T cells isolated from oligomeric IgD-treated mice and control mice were washed extensively following staining with biotinylated IgD (0.5 μg/10^6 cells) in order to examine the binding affinity of IgD-R. FACS analysis indicated that the biotinylated IgD bound to IgD-R on Tδ cells was stronger as compared with those found on control T cells, and showed that 43% of control T cells and 56% of Tδ cells were IgD-R-positive. Alternatively, incubation of T cells with various doses of unconjugated IgD prior to staining with biotinylated-

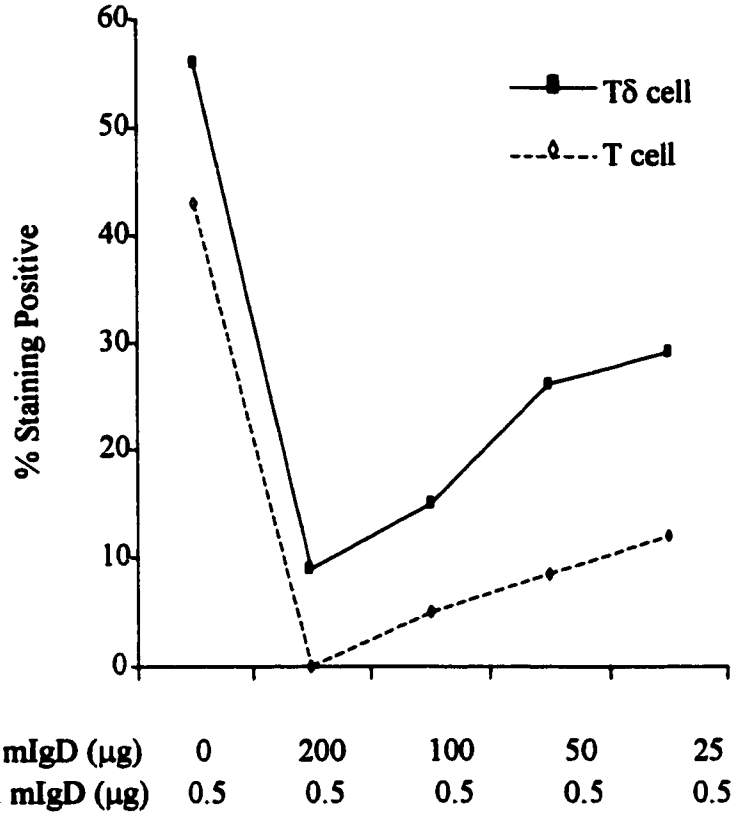

Figure 3. ***Dose dependent inhibition of IgD-R and IgD interaction.*** BALB/c mice were primed i.v. on day -1 with oligomeric IgD (100 μg) or with saline. Tδ cells and T control cells, respectively, were isolated and stained with biotinylated-IgD (0.5 μg/1x10^6 cells) in the presence or absence of increasing concentrations of unconjugated IgD. Samples were analyzed by FACS. 20,000 T cells were analyzed based on light scatter properties. Results shown are representative of 3 experiments performed ($\rho<0.015$). Avidin-FITC yielded backgrounds have been subtracted,

IgD, showed that binding of biotinylated-IgD to T cells was blocked by unconjugated IgD in a dose-dependent manner. As seen in figure 3, incubation with unconjugated IgD at the dose 200 μg, 100 μg, 50 μg and 25 μg showed that 0%, 5%, 8% and 12%, respectively, of control T cells stained positive with biotinylated IgD, and 9%, 15%, 26% and 29%, respectively, of T cells previously induced with oligomeric IgD (Tδ cells) stained positive. Compared with normal staining (43% positive of control T cells and 56% positive of Tδ cells), unconjugated IgD gave blocked 100%, 88%, 80% and 70% at the doses tested using control T cells and 83%, 73%, 48% and 46% blockade using Tδ cells. Thus, the competitive inhibition of unconjugated IgD (IgD-R blocking effects) on control T cells were 20% higher than blocking effects on Tδ cells. The data indicates that oligomeric IgD of treatment of normal T cells leads not only to increased expression of IgD-R, but also to an increase in the IgD binding affinity of IgD-R on these cells.

4. *Oligomeric IgD treatment in vivo enhances primary antibody responses to SRBC as measured in direct plaque assays.*

The effects of treatment with oligomericIgD *in vivo* on primary antibody response can be seen in Figure 4. In this experiment, BALB/c mice were immunized i.v. on day -5 with $1x10^8$ SRBC alone (as control), or together with 100 μg of oligomeric IgD. Whole spleen cells from control or IgD-treated mice were used to compare antibody response to SRBC. The primary antibody responses were measured as anti-SRBC plaque forming cells (PFCs) as described in materials and methods. As shown in figure 4, the number of specific anti-SRBC plaque forming cells were significantly increased from 1700 ± 253 in control cells to 3100 ± 521 in IgD-induced cells ($\rho<0.01$). The study also found that oligomeric IgD pretreatment *in vivo* augmented the

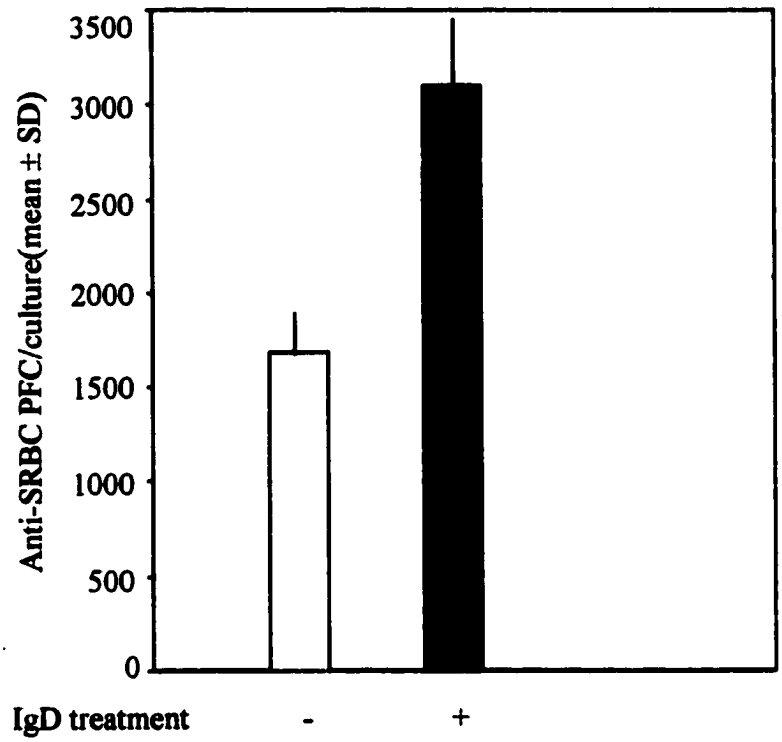

Figure 4. ***Oligomeric IgD treatment in vivo enhances primary antibody response to SRBC as measured in direct plaque assay.*** BALB/c mice were immunized i.v. on day -5 with $1x10^8$ SRBC, with or without 100 μg oligomeric IgD (n=5 per group). Spleen cells from control or IgD treated mice were assayed for SRBC-specific PFC responses as described in Materials and Methods on day 0. Results are expressed as the mean number of anti-SRBC PFC per spleen.

secondary antibody response only when it was injected simultaneously with the primary antigen injection.(Yang, 1996; Swenson, 1995).

5. *IgD pre-treatment in vivo enhances in vitro primary antibody response to SRBC as measured in direct plaque assay.*

In this study, we examined whether IgD pretreatment in vivo would predispose spleen cells to mount further enhanced antibody responses *in vitro*. The effects of IgD pretreatment on primary antibody responses generated *in vitro* can be seen in figure 5. In this study, BALB/c mice were injected with 100 μg of oligomeric IgD three days prior to the establishment of spleen cell cultures *in vitro*. Following *in vivo* treatment, spleen cells were isolated and cultured *in vitro* for three more days in presence of SRBC. The antibody responses were measured as anti-SRBC plaque forming cells. As shown in figure 5, primary anti-SRBC PFC responses generated *in vitro* were significantly enhanced (3805 ± 465) as compare with those seen in cultures of spleen cells derived from control mice (1804 ± 244) ($\rho<0.001$).

6. *IgD pre-treatment in vivo enhances in vitro secondary antibody response to SRBC as measured in direct plaque assay.*

Oligomeric IgD treatment *in vivo* significantly enhanced primary antibody response generated *in vitro*, as shown in figure 5. Similar effects of oligomeric IgD were also observed in early secondary anti-SRBC response generated *in vitro*. Briefly, 1×10^8 SRBC alone (as control) or with 100 μg of oligomeric IgD were injected i.v. on day 0 into BALB/c mice whose spleen cells were then harvested after 3 days and were cultured for 72 hours in the presence of SRBC.

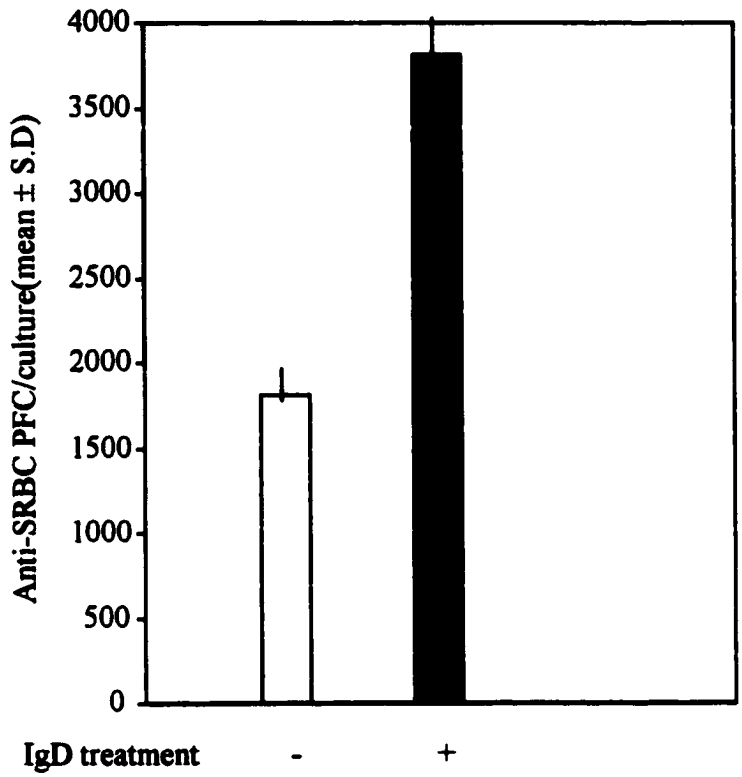

Figure 5. ***IgD pre-treatment in vivo enhances in vitro primary antibody response to SRBC as measured in direct plaque assay.*** BALB/c mice were immunized i.v. on day -3 with or without 100 μg oligomeric IgD (n=5 per group). Spleen cells from control or IgD pre-treated mice were cultured on day 0 at $5x10^6$/ml in the presence of 50 μl/ml of 0.1% SRBC (SRBC-primed cells) for 3 days and then assayed for SRBC-specific PFC responses. Results are expressed as the mean number of anti-SRBC PFC per spleen.

The antibody responses were measured as anti-SRBC plaque forming cells (PFC) as described in materials and methods. As shown in figure 6, secondary anti-SRBC PFC responses were significantly enhanced as compared with those seen in cultures of spleen cells derived from control mice ($\rho<0.05$). The number of anti-SRBC specific plaque forming cells were significantly increased from 2218 ± 295 in control cells to 4780 ± 541 in cells previously exposed to oligomeric IgD *in vivo*. The data consistent with result from previous studies (Yang, 1996).

7. *IgD pretreatment in vivo enhances primary and secondary antibody responses to SRBC as measured in ELISPOT assays.*

In this and previous studies, we have relied on a plaque assay technique to quantitate antibody responses. In the current studies, a new improved technique, namely, the ELISPOT assay, was also used to measure antibody forming cells. Mice were primed i.v. with a single dose of 1×10^8 SRBC alone (as control) or together with purified oligomeric IgD (100 μg) 5 days before ELISPOT assays were performed and primary antibody responses were measured according to the method described by Coico and Licke (1996). As shown in figure 7A and 7B, the numbers of primary anti-SRBC antibody forming cells were significantly increased as compared with those seen in cultures of spleen cells derived from control mice. Particularly when 10% SRBC lysate was used to coat the membrane, minimal background and maximal number of Ab-forming cells were observed. As shown table 1, coating the membrane with a 10% SRBC lysate was optimum for measuring primary antibody responses (2204 ± 284) as compared with the 7.5% (1130 ± 360) or 15% (2053 ± 327) lysates tested ($\rho<0.001$). Similar

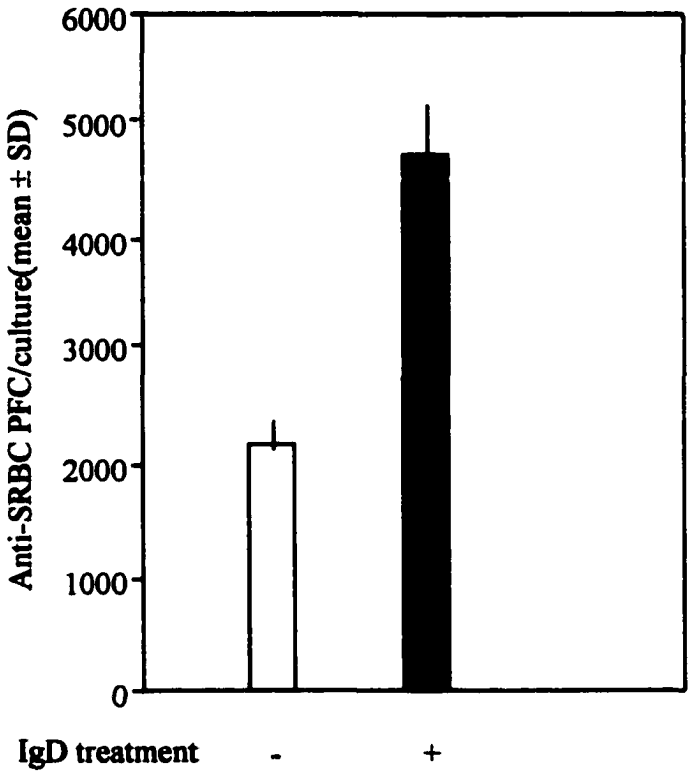

Figure 6. ***IgD pre-treatment in vivo enhances in vitro early secondary antibody response to SRBC as measured in direct plaque assay.*** BALB/c mice were immunized i.v. on day -3 with $1x10^8$ SRBC alone (as control) (n=5 per group), or together with 100 μg oligomeric IgD. Spleen cells from control or IgD pre-treated mice were cultured on day 0 at $5x10^6$/ml in the presence of 50 μl/ml of 0.1%SRBC (SRBC-primed cells) for 3 days and then assayed for SRBC-specific PFC responses. Results are expressed as the mean number of anti-SRBC PFC per spleen.

effects of oligomeric IgD were observed in measuring secondary anti-SRBC antibody responses. Mice were primed i.v. with 1 x 10^8 SRBC on day-14 alone (as control) or together with purified oligomeric IgD (100 μg), followed by an i.v. boost of 1 x 10^8 SRBC on day -5 and ELISPOT assays were performed on day 0 to measure the secondary antibody responses. As shown in figure 8A and 8B, the numbers of secondary anti-SRBC antibody forming cells were significantly increased from IgD treated mice as compared with control mice ($\rho<0.001$). Again, the 10% SRBC lysate was optimum for measuring ELISPOTs (6500±628) as compared with the 7.5% (3750±561) and 15% (5304±613) lysates tested (table 1). Taken together, these data show that IgD-R^+ T cells enhance antibody responses in B cells and suggest that IgD-R and IgD interactions play important role in enhancing both primary and especially secondary antibody responses.

8. *IgD pretreatment in vivo enhances primary and secondary antibody responses to TNP-OVA as measured in ELISPOT assays.*

We have also utilized another antigen, namely TNP-ovalbumin (TNP-OVA) to examine the immunoregulatory effects of IgD. Results of ELISPOT assays examining antibody responses to TNP-OVA immunizations administered together with IgD were similar to those obtained using SRBC immunizations and showed enhancement anti-TNP antibody responses (figures 7 and 8). Details of the experimental design are given in the legends of figures 7 and 8 as well as in the general materials and methods section. As shown in figures 9 and 10 and table 2, antibody responses are significantly enhanced by simultaneous treatment with TNP-OVA and

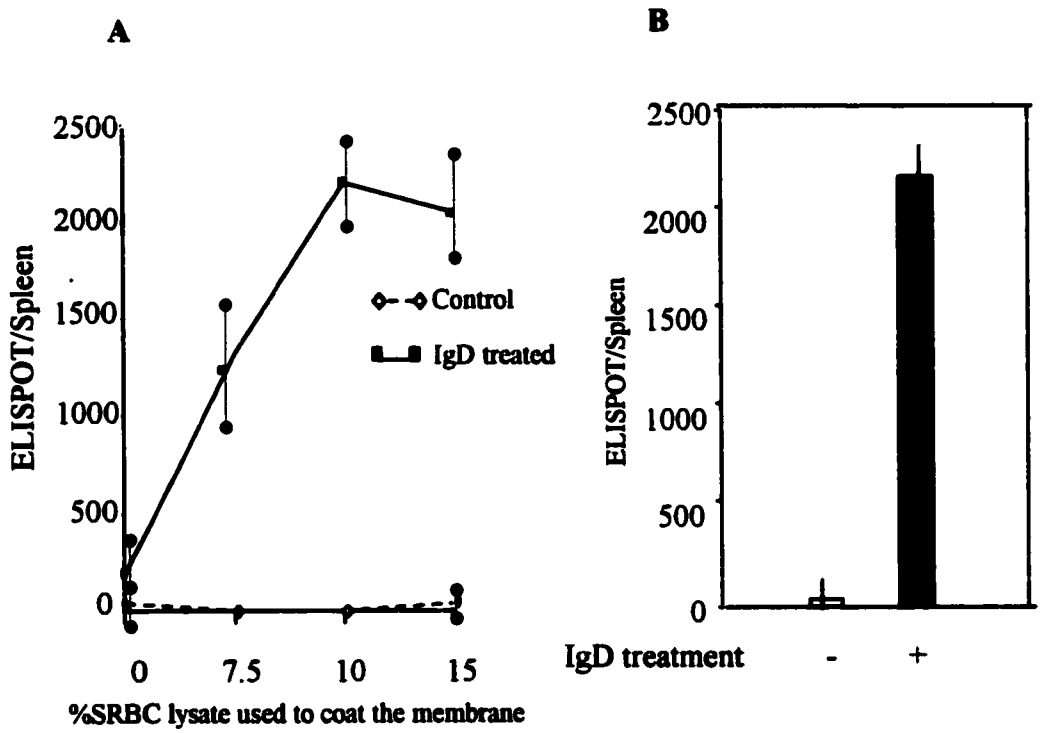

Figure 7. ***IgD pre-treatment in vivo enhances primary antibody responses to SRBC as measured in ELISPOT assays.*** BALB/c mice were immunized i.v. with $1x10^8$ SRBC on day -5, with or without 100 μg of oligomeric IgD (n=5/group). (A) ELISPOT assays with spleen cells using various percentages of SRBC lysate-coated membranes were performed on day 0. Results are expressed as the mean number of anti-SRBC ELISPOT per spleen by subtracting BSA-coated membrane-yielded backgrounds. (B) Primary responses to SRBC measured in ELISPOT assays by using 10%SRBC lysate-coated membrane.

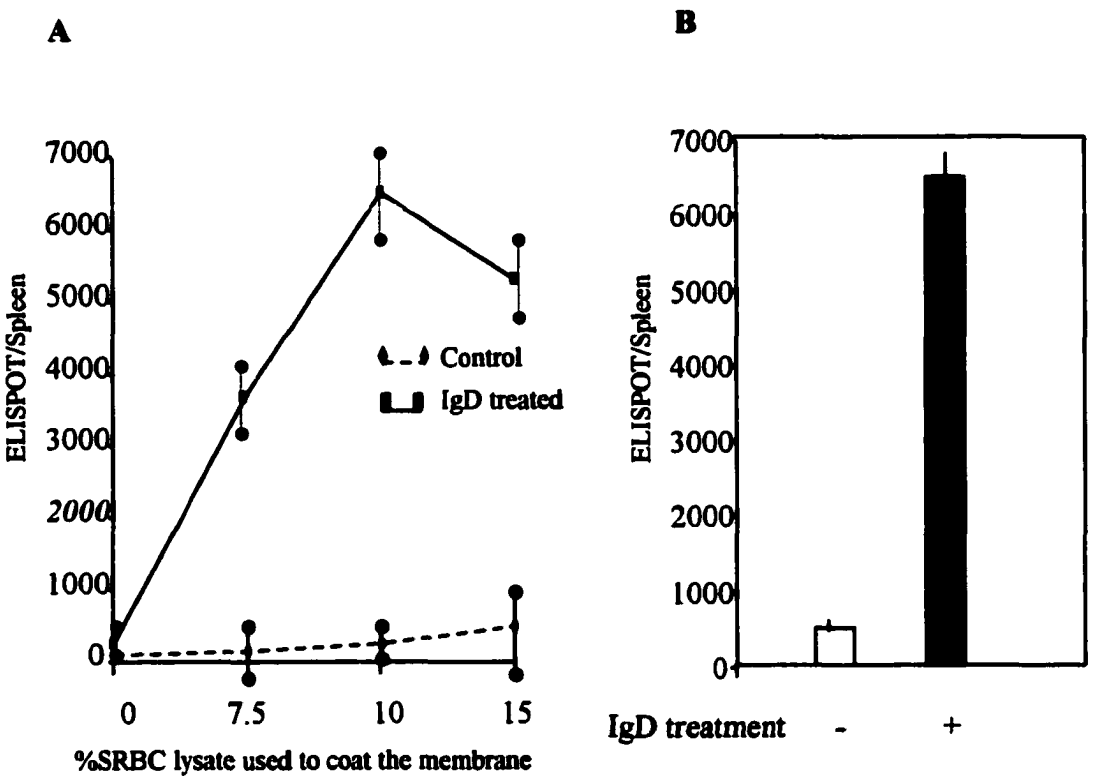

Figure 8. ***IgD pretreatment in vivo enhances secondary response to SRBC as measured in ELISPOT assays.*** BALB/c mice were immunized i.v. with $1x10^8$ SRBC on day -14, with or without 100μg of oligomeric IgD (n=5/group), followed by an i.v. boost of $1x10^8$SRBC on day -5. (A). ELISPOT assays with spleen cells using various percentages of SRBC lysate-coated membranes were performed on day 0. Results are expressed as the mean number of anti-SRBCELISPOT per spleen by subtracting BSA-coated membrane-yielded background. (B). Secondary responses to SRBC measured in ELISPOT assays by using 10%SRBC lysate-coated membrane.

Table 1. *In vivo* treatment with oligomeric IgD augments SRBC-specific primary and secondary antibody responses

		Anti-SRBC ELISPOT/Spleen (mean ± SD)	
SC [a] or SCδ [b]	%SRBC lysate used to coat the membrane	primary antibody responses [c]	secondary antibody responses [d]
SC	0	40±80	100±70
SC	7.5	0	150±122
SC	10	0	250±70
SC	15	40±80	500±187
SCδ	0	190±120	250±70
SCδ	7.5	1330±360	3750±561
SCδ	10	2204±284	6500±628
SCδ	15	2053±327	5304±613

[a] Spleen cells were harvested from BALB/c mice immunized i.v. with $1x10^8$ SRBC alone.

[b] Spleen cells were harvested from BALB/c mice immunized i.v. with $1x10^8$ SRBC together with 100 μg of oligomeric IgD.

[c] To analyze primary antibody responses, BALB/c mice were immunized i.v. with $1x10^8$ SRBC on day -5, with or without 100 μg of oligomeric IgD.

[d] To analyze secondary antibody responses, BALB/c mice were immunized i.v. with $1x10^8$ SRBC on day -14, with or without 100 μg of oligomeric IgD, followed by an i.v. boost of $1x10^8$ SRBC on day -5.

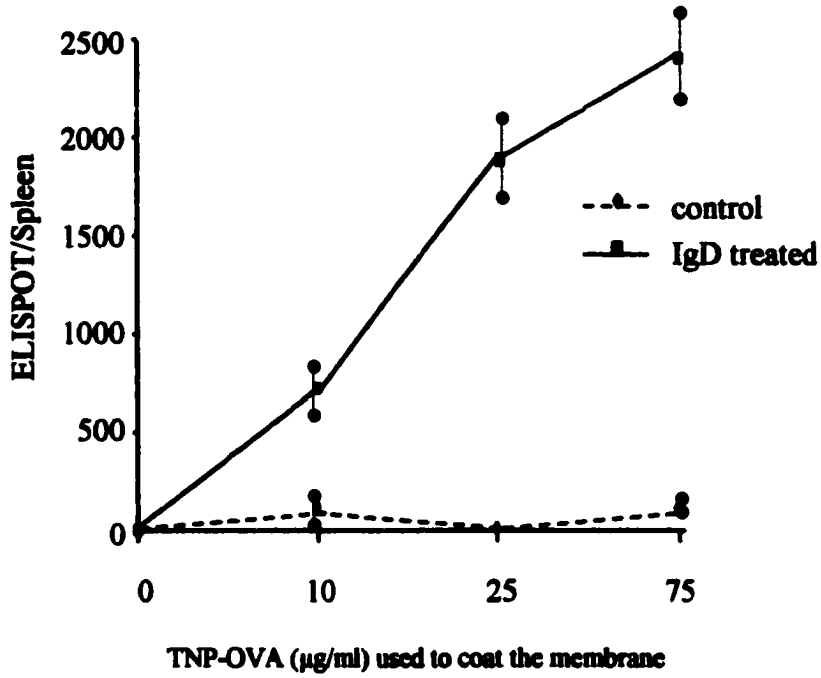

Figure 9. ***IgD pretreatment in vivo enhances primary antibody responses to TNP-OVA as measured in ELISPOT assays.*** **Anti-TNP ELISPOTS were measured using different amounts of TNP-OVA to coat membranes. BALB/c mice were immunized i.v. with 80 μg of TNP-OVA on day -5 with or without 100 μg of oligomeric IgD (n=5/group). Spleen cell ELISPOT assays were performed on day 0. BSA-coated membrane-yielded backgrounds have been subtracted. Results are expressed as the mean number of anti-TNP-OVA ELISPOT per spleen.**

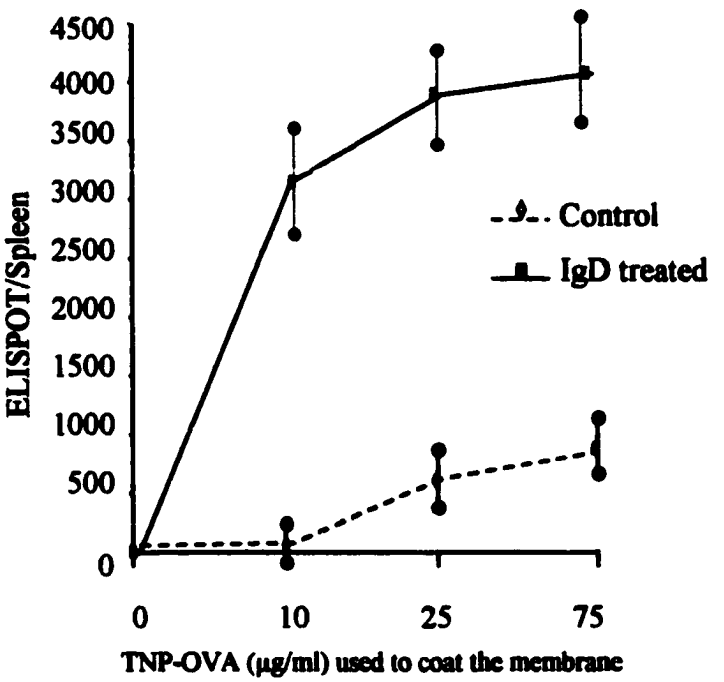

Figure 10. ***IgD pretreatment in vivo enhances secondary antibody responses to TNP-OVA as measured in ELISPOT assays.*** BALB/c mice were immunized i.v. with 80 μg of TNP-OVA on day -14, with or without 100 μg of oligomeric IgD, followed by an i.v. boost of 80 μg of TNP-OVA on day –5 (n=5/group). ELISPOT assays were performed on day 0 with spleen cells by using various doses of TNP-OVA to coat membranes. BSA-coated membrane-yielded backgrounds have been subtracted. Results are expressed as the mean number of anti-TNP-OVA ELISPOT per spleen.

Table 2. *In vivo* treatment with oligomeric IgD augments TNP-OVA- specific primary and secondary antibody responses

		Anti-TNP-OVA ELISPOT/Spleen (mean ± SD)	
SC [a] or SCδ [b]	TNP-OVA used to coat the membrane (μg/ml)	primary antibody responses [c]	secondary antibody responses [d]
SC	0	0	0
SC	10	25±40	25±40
SC	25	0	569±146
SC	75	50±1	787±152
SCδ	0	0	0
SCδ	10	720±117	3150±536
SCδ	25	1920±296	3900±458
SCδ	75	2400±306	4080±541

[a] Spleen cells harvested from BALB/c mice immunized i.v. with $1x10^8$ SRBC alone.

[b] Spleen cells harvested from BALB/c mice immunized i.v. with $1x10^8$ SRBC together with 100 μg of oligomeric IgD.

[c] To analyze primary antibody responses, BALB/c mice were immunized i.v. with 80 μg of TNP-OVA on day -5, with or without 100 μg of oligomeric IgD.

[d] To analyze secondary antibody responses, BALB/c mice were immunized i.v. with 80 μg of TNP-OVA on day -14, with or without 100 μg of oligomeric IgD, followed by an i.v. boost of 80μg TNP-OVA on day -5.

oligomeric IgD as compared with responses to TNP-OVA alone (control) ($\rho<0.01$). Furthermore, these increased antibody responses are dose dependent.

9. *Comparision of purification of T cells or B cells by immunomagnetic isolation or panning.*

Highly purified T and B lymphocytes are essential to analyze their functional capacity, as the presence of other cell types can alter cell functions by surface interaction or by production of cytokines. There are a varity of available techniques for lymphocyte isolation, many of which are based on antibody-mediated selection-e.g., panning of immobilized antibody, antibody /complement-mediated lysis, cell sorting of fluorescence-labeled cells and immunomagnetic selection. Immunomagnetic separation was a new technique used in our laboratory at the initiation of these studies. Its advantages over other antibody-mediated selection techniques are purity of resulting cell preparation, reproducibility of separation, and ease of handling small to large numbers of cells (10^6 to10^{10} cells). Using this technique, splenic T cells were negatively selected by depletion of B cells with goat anti-mouse Ig-coupled magnetic microbeads. Purified T cells were washed and checked for purity. Staining with anti-CD3-FITC and B220-FITC indicated that 90% of these isolated cells were CD3 positive T cells, with only 2% contamination by B220 positive B cells as shown in figure 11, E-F. We compared immunomagnetic separation of T and B lymphocytes with traditional panning technique (figure 11, H-I). Briefly, T cells were prepared by depletion of adherent cells at 37°C for 1 hour on tissue culture grade Petri dishes and of B cells by panning spleen cells on Petri dishes coated with affinity-purified goat anti-mouse IgG+IgM (Neuberger et . al ., 1981). Purified T cells were washed and checked for purity. FACS analysis of anti-CD3-FITC and B220-FITC staining showed that 80% of the

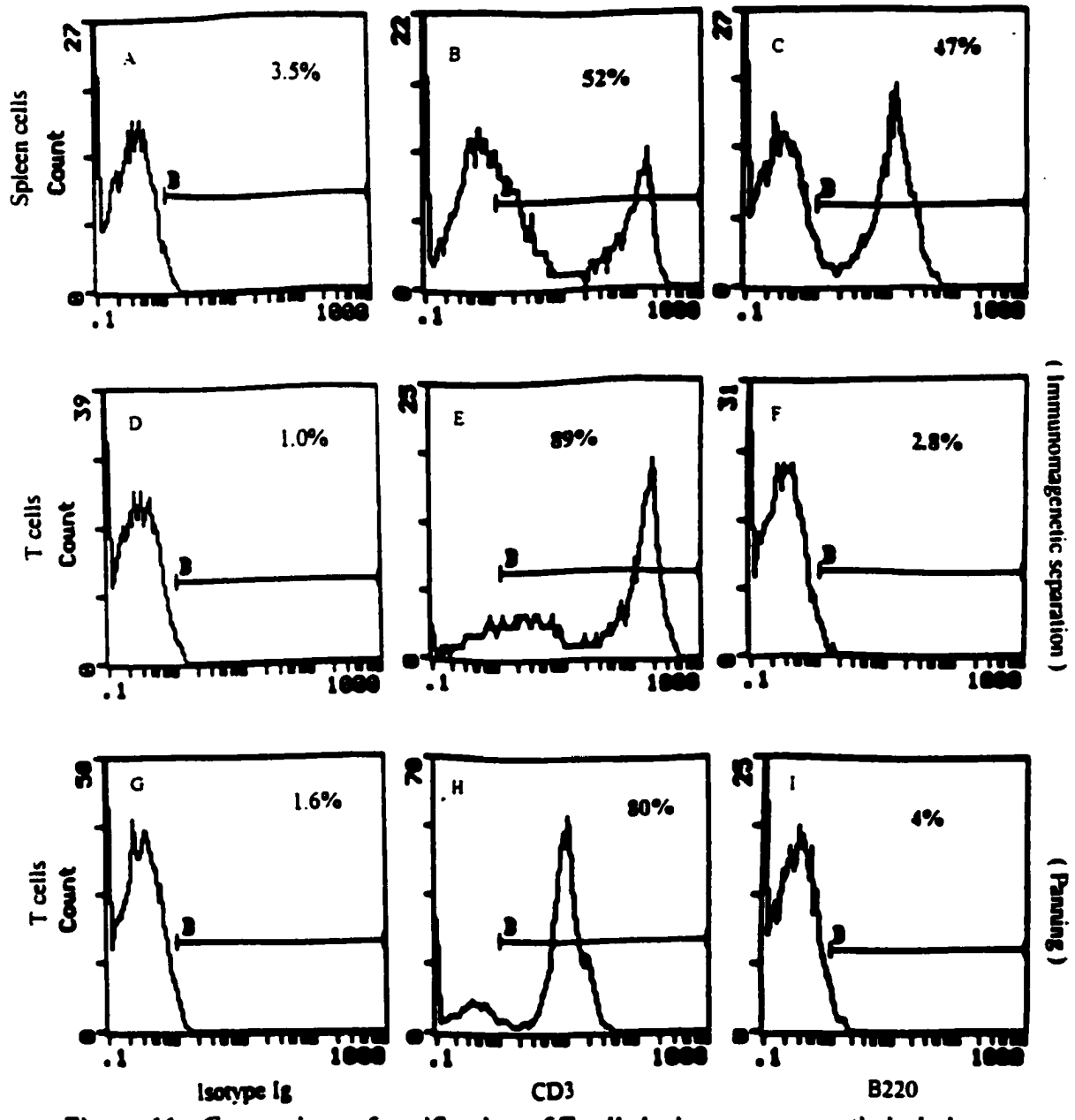

Figure 11. ***Comparison of purification of T cells by immunomagnetic isolation or panning.*** $1x10^7$ spleen cells were incubated with anti-Ig-coated microbeads at 4°C for 45 minutes, then T cells were isolated by the immunomagnetic separation columns. Cells were stained with isotype Ig-FITC, or anti-CD3-FITC and B220-FITC and analyzed by flow cytometry (D,E,F). Alternatively, $3x10^7$ spleen cells depleted of adherent cells (e.g. macrophages) were panned on goat anti-mouse IgG+IgM coated dishes at 4°C for 1.5 h, and the non-adherent cells were harvested. Then cells were stained with isotype Ig-FITC, or anti-CD3-FITC and B220-FITC and analyzed by flow cytometry (G,H,I). Results shown are representative of 5 experiments performed. Whole spleen cells staining with isotype Ig, or anti-CD3-FITC and B220-FITC served as control (A,B,C).

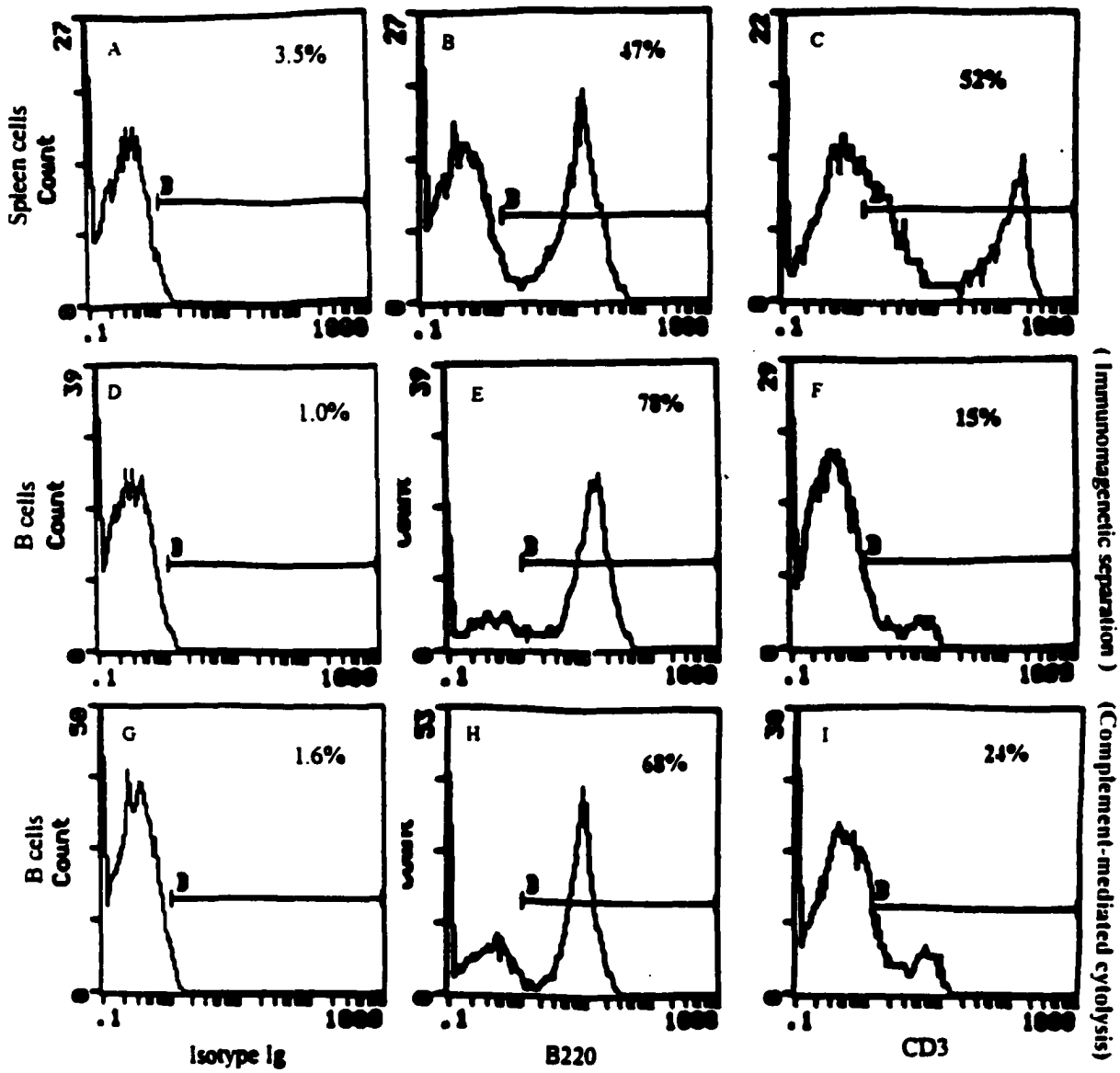

Figure 12. ***Comparison of purification of B cells by immunomagnetic isolation or complement-mediated cytolysis.*** $1x10^7$ spleen cells were incubated with anti-Thy1.2-coated microbeads at 4°C for 45 minutes, then B cells were isolated by the immunomagnetic separation and stained with isotype Ig-FITC, or B220-FITC and anti-CD3-FITC and analyzed by flow cytometry (D,E,F). Alternatively, $1x10^7$spleen cells were incubated with anti-Thy1.2 (diluted to 1:20) on ice for 1 h. then rabbit complement was added and cells were incubated at 37°C water bath for additional 1 h to lyse the anti-Thy1.2 coated T cells. Then cells were stained with isotype Ig-FITC, or B220-FITC and anti-CD3-FITC and analyzed by flow cytometry (G,H,I). Results shown are representative of 5 experiment performed. Whole spleen cells staining with isotype Ig, or B220-FITC and anti-CD3-FITC served as control (A,B,C).

isolated cells were CD3 positive T cells, and 4% were contaminating B220 positive B cells , as shown in figure 11, H-I. These data indicated that even though both methods provide purity of cells, but magnetic separation provided slightly improved purity of T lymphocytes. Similarly, B cells were isolated by immunomagnetic separation with depletion of T cells by anti-Thy1.2 magnetic microbeads. Purified B cells were washed and checked for purity. Staining with B220-FITC and anti-CD3-FITC indicated that 80% of the isolated cells were B220 positive B cells, and 14% were contaminating CD3 positive T cells, as shown in figure 12, E-F. However, when B cells were isolated by antibody /complement mediated cytolytic elimination of T cells, FACS analysis showed that 70% of the isolated cells were B220 positive B cells, and 22% were contaminating CD3 positive T cells, as shown in figure 12, H-I. Sequential immunomagnetic separation of T and B cells results in >95% purity of these populations. The data indicates that immunomagnetic selection increases the purity of resulting cells preparation as compare with other techniques, moreover, the cells recovery also increased by approximately 10-15%.

10. *B cells serve as highly efficient antigen presenting cells to antigen-primed T cells expressing IgD-R.*

Goat Ig-primed T cells were isolated from the spleens of control BALB/c mice or mice treated with oligomeric IgD to induce IgD-R expression in vivo. Peritoneal addherent cells and splenic B cells purified from control BALB/c mice were pulsed with GAM Ig, irradiated and used as APC to assess the responses of goat Ig-primed T cells and Tδ cells. As shown in figure 13, when GAM Ig-pulsed and irradiated adherent cells were used as APC, similar proliferative responses were seen when comparing goat Ig-primed control T versus Tδ cells. When GAM Ig

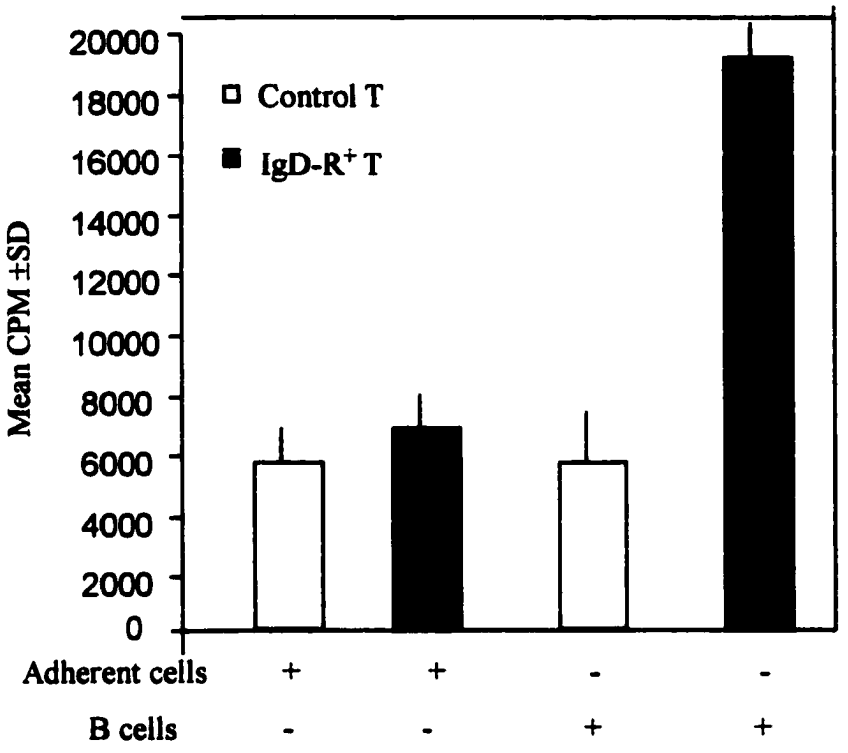

Figure13. ***B cells serve as highly efficient antigen presenting cells to antigen-primed T cells expressing IgD-R (Tδcells).*** Peritoneal adherent cells and or splenic B cells were purified from BALB/c mice, pulsed with 10 μg/ml GAM-Ig, irradiated and used as APCs at a density of $1x10^5$ cells/well. Responding T cells were isolated from BALB/c mice primed in vivo with goat Ig alone (control T) or from mice primed with goat Ig and with oligomeric IgD (50 μg) on day -1 (IgD-R^+T). Following a 72h culture period, cells were labeled with 1μCi ^{3}H-TdR for an additional 6 h, harvested and analyzed by scintillation counting. Results shown are representative of 3 experiments performed.

pulsed B cells were used as APC, proliferative responses of goat Ig-primed Tδ cells were significantly greater ($P<0.05$) than those of control T cells isolated from goat Ig-primed mice. In contrast, antigen-presenting adherent cells promote similar levels of responses by T cells regardless of whether the responding cells have been induced to express IgD-R (figure 14).

We next compared the responses to goat Ig-primed T and Tδ cells to B cells presenting GAM IgD or GAM IgM in an effort to determine whether crosslinking of specific B cell membrane Igs had any influence on the enhanced responses of Tδ cells. As shown in figure 15, antigen-primed Tδ cells responded slightly more efficiently to GAM-IgM sensitized B cells as compared with GAM-IgD sensitized B cells, although the differences are not significant ($P>0.05$). Consistent with results shown in figure 13, responses of goat Ig-primed control T cells were dramatically lower than those of Ag-primed Tδ cells suggesting that expression of IgD-R in the latter enables such cells to respond more efficiently to antigen presented by B cells. Taken together, these results indicate that B cells are more efficient as APCs when responding Ag-primed T cells are previously induced to express IgD-R.

11. *Monomeric IgD (KWD9) blocks antigen presentation by goat Ig-primed T and Tδ cells stimulated with or without GAM IgM-treated B cells.*

We have previously shown that monomeric IgD fails to induce IgD-R expression by $CD4^+$ T cells although monomeric forms of IgD can inhibit the binding of IgD-R to IgD as measured in rosetting assays (Coico et. al. 1988) and in immunofluoresent staining (figure 2). Monomeric forms of IgD also interfere with in vivo and in vitro effects of Tδ cells with regard to their enhancement of antibody responses (Swenson et al.1995). In the present study, we asked

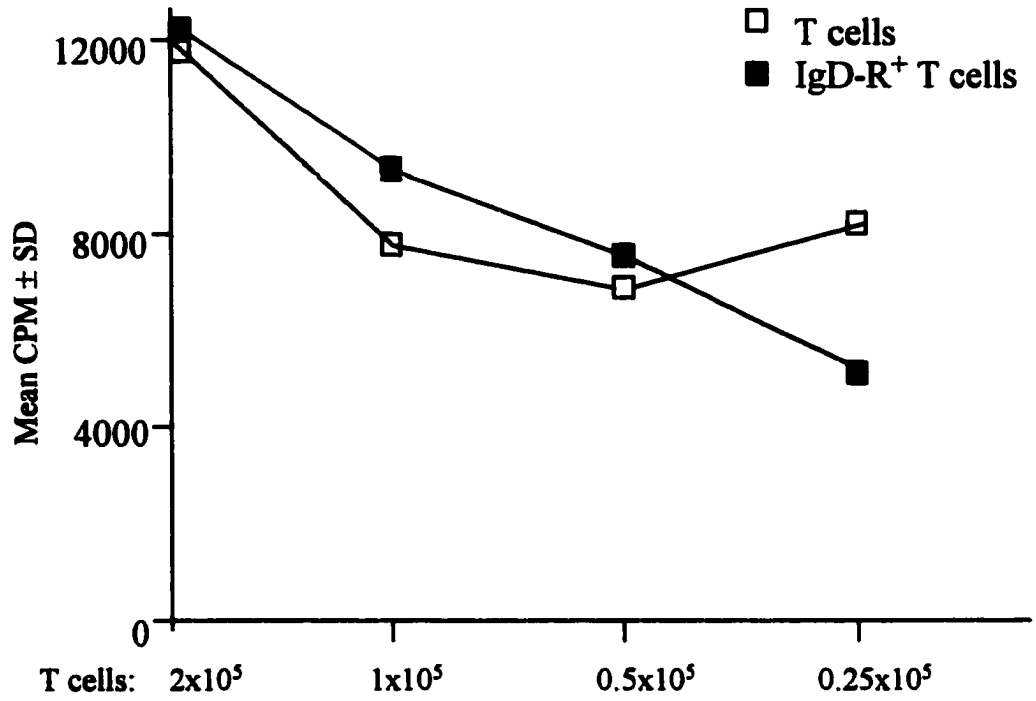

Figure 14. ***Goat Ig-primed T cells and IgD-R$^+$Tδ cells respond similarly when GAM-Ig pulsed adherent cells are used as APCs.*** Adherent peritoneal cells were prepared from BALB/c mice as described in Materials and Methods, pulsed with 10μg/ml GAM-Ig, irradiated, and used as APCs. APCs were then co-cultured with goat Ig-primed splenic T cells and IgD-R$^+$Tδ cells induced as described in figure 13 at the cell numbers indicated. Following a 72 h culture period, cells were labeled with 1μCi ^{3}H-TdR for an additional 6 h, harvested and analyzed by scintillation counting. Results shown are representative of 3 experiments performed.

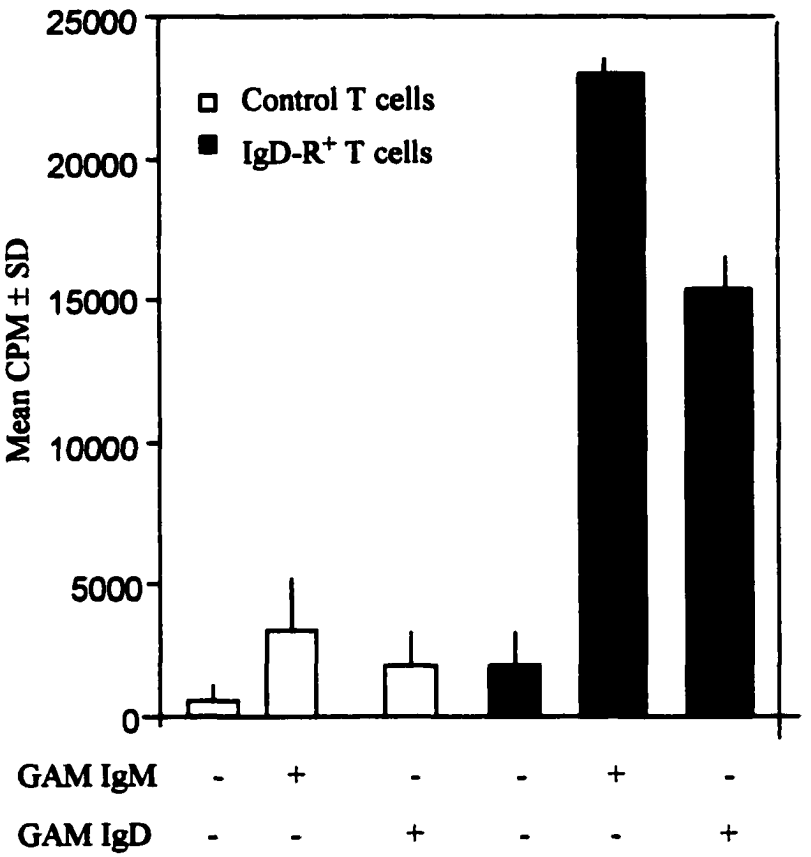

Figure 15. ***Comparison of goat-Ig primed T and Tδ cells responses stimulated by B cells presenting GAM IgM vs GAM IgD.*** Splenic B cells were purified from BALB/c mice, pulsed with or without 10μg/ml GAM IgM or GAM IgD, irradiated, and used as APCs at density of $1x10^5$cells/well. APCs were then co-cultured with goat Ig-primed splenic T cells and IgD-R^+ Tδ cells induced as described in figure 13 at the density of $1x10^5$ cells/well. Following a 72 h culture period,cells were labeled with 1μCi ^{3}H-TdR for an additional 6 h, harvested and analyzed by scintillation counting. Results shown are representative of 3 experiments performed.

whether the addition of monomeric IgD to cultures of T and B cells would inhibit the enhanced T cell responses seen when T cells are previously induced to express IgD-R (Figure D). As shown in figure 16, monomeric IgD (KWD9) blocked responses of goat Ig-primed Tδ cells in a dose-dependent manner when these cells were stimulated with B cells pulsed with 10 μg/ ml of GAM IgM. The result show that when 1, 10, 100 μg/ ml of KWD9 were added to cultures, a 22%, 62%, and 85% inhibition, respectively, was obtained.

We also examined the inhibitory effects of various forms of monomeric IgD on IgD-R-IgD interactions and subsequent T-B interaction. A similar inhibition was observed with the other monomeric IgDs such as KWD8, KWD1 and B1.8δ. The results are shown in figures 17, 18 and 19; and the efficiency of inhibition by KWD1 and B1.8δ on Tδ cell responses were lower than that observed using the KWD8 and KWD9 monomeric IgDs. The different levels of inhibition by these monomeric IgDs on T and Tδ cells response can be seen in table 3. Interestingly, these monomeric forms of IgD also suppressed responses of Ag-primed control T cells suggesting that the basal level of IgD-R present on such cells may also contribute to cognate T-B interactions during antigen presentation.

12. *IgD-R can be upregulated on T cells from IgD-KO mice follwoing in vivo or in vitro treatment with oligomeric IgD.*

Although the functional activity of oligomeric IgD is absent in $IgD^{-/-}$ mice, Tδ cells with upregulated IgD-R have been observed in $IgD^{-/-}$ mice if they received IgD treatment *in vivo* or *in vitro* (Swenson et al., 1995). Following IgD treatment *in vivo* or *in vitro*, IgD receptors have seen to be upregulated at levels similar to those seen as in T cells from $IgD^{+/-}$ mice. As shown in

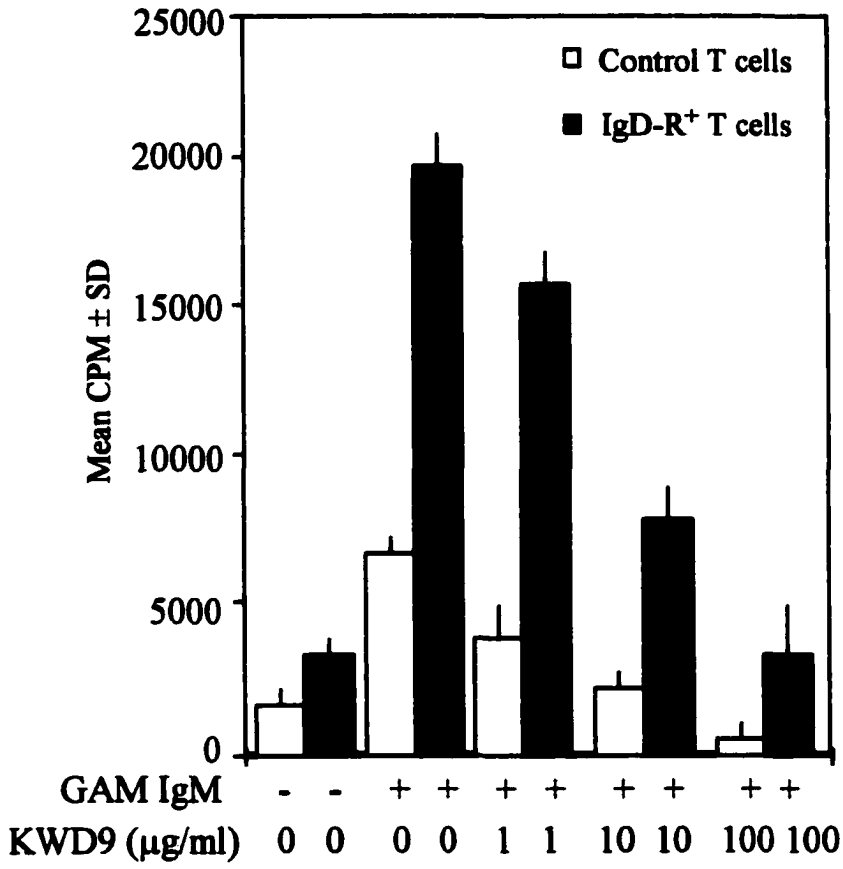

Figure 16. ***Monomeric IgD KWD9 inhibits antigen presentation by goat Ig -primed T and Tδ cells stimulated with or without GAM IgM-treated B cells.*** Purified splenic B cells from BALB/c mice at a density of $1x10^5$ cells/well were pulsed with or without 10 μg/ml GAM IgM, irradiated, then co-cultured with goat Ig-primed splenic T cells and IgD-R^+ Tδ cells induced as described in earlier at the density of $1x10^5$ cells/well. Following a 72 h culture period, cells were labeled with 1μCi ^{3}H-TdR for an additional 6 h, harvested and analyzed by scintillation counting. Results shown are representative of 5 experiments performed. Culture of purified T and B cells in absence of 10 μg/ml GAM IgM served as control.

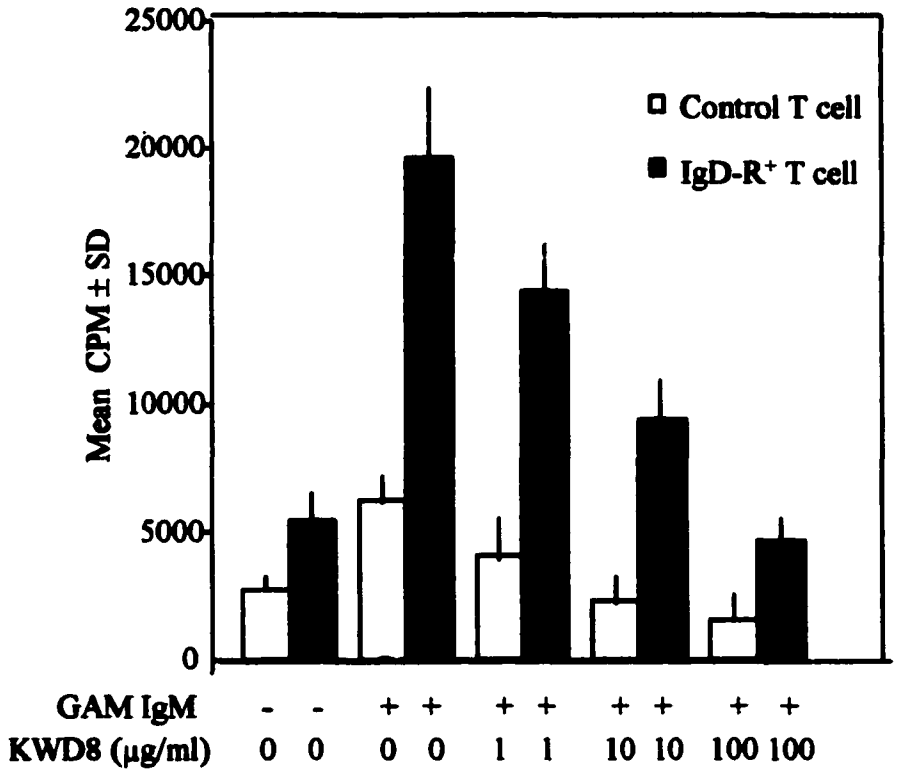

Figure 17. ***Monomeric IgD KWD8 inhibits antigen presentation by goat Ig -primed T and Tδ cells stimulated with or without GAM IgM-treated B cells.*** Purified splenic B cells from BALB/c mice at a density of $1x10^5$cells/well were pulsed with or without 10μg/ml GAM IgM, irradiated, then co-cultured with goat Ig-primed splenic T cells and IgD-R^+ Tδ cells induced as described in figure 13 at the density of $1x10^5$ cells/well. Following a 72 h culture period,cells were labeled with 1μCi ^{3}H-TdR for an additional 6 h, harvested and analyzed by scintillation counting. Results shown are representative of 5 experiments performed. Culture of purified T and B cells in absence of 10 μg/ml GAM IgM served as control.

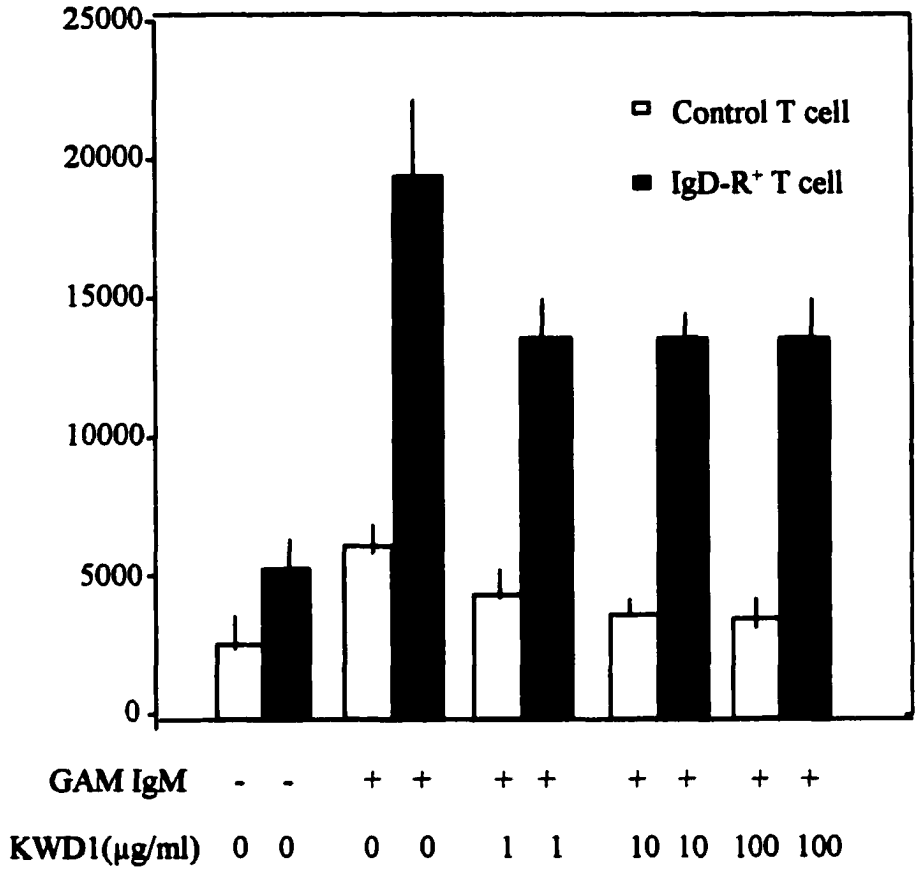

Figure 18. ***Monomeric IgD KWD1 inhibits antigen presentation by goat Ig -primed T and Tδ cells stimulated with or without GAM IgM-treated B cells.*** Purified splenic B cells from BALB/c mice at a density of $1x10^5$cells/well were pulsed with or without 10μg/ml GAM IgM, irradiated, then co-cultured with goat Ig-primed splenic T cells and IgD-R^+ Tδ cells induced as described in figure 13 at the density of $1x10^5$ cells/well. Following a 72 h culture period,cells were labeled with 1μCi ^{3}H-TdR for an additional 6 h, harvested and analyzed by scintillation counting. Results shown are representative of 5 experiments performed. Culture of purified T and B cells in absence of 10μg/ml GAM IgM served as control.

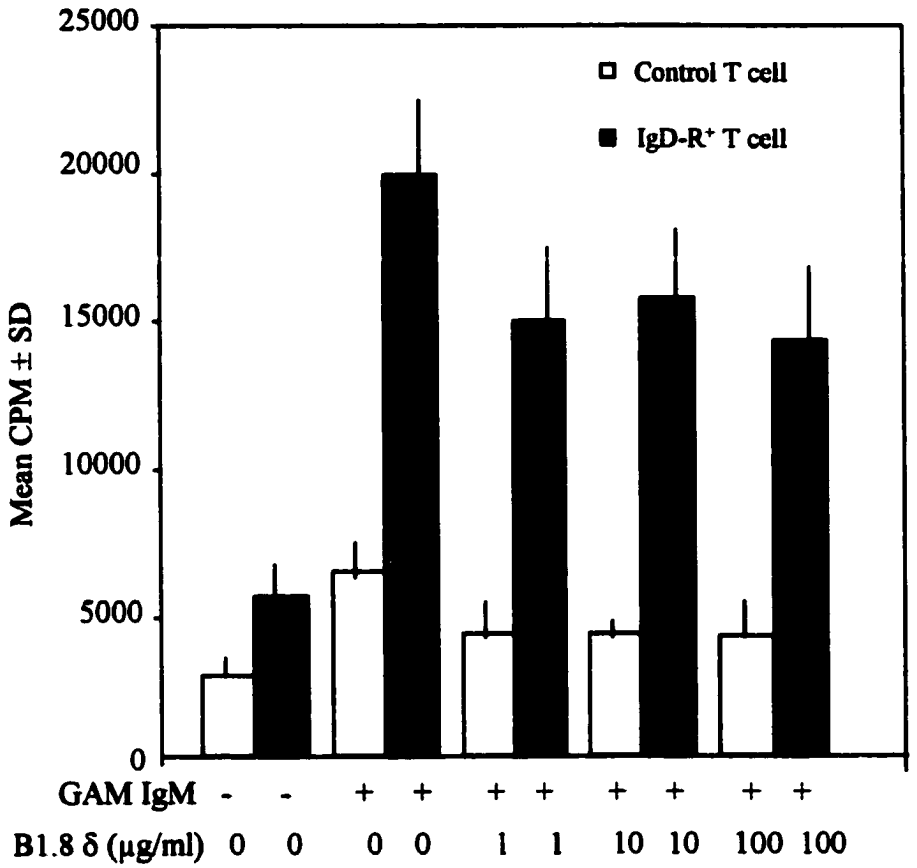

Figure 19. ***Monomeric IgD B1.8δ inhibits antigen presentation by goat Ig -primed T and Tδ cells stimulated with or without GAM IgM-treated B cells.*** Purified splenic B cells from BALB/c mice at a density of $1x10^5$ cells/well were pulsed with or without 10μg/ml GAM IgM, irradiated, then co-cultured with goat Ig-primed splenic T cells and IgD-R^+ Tδ cells induced as described in figure 13 at the density of $1x10^5$ cells/well. Following a 72 h culture period,cells were labeled with 1μCi ^{3}H-TdR for an additional 6 h, harvested and analyzed by scintillation counting. Results shown are representative of 5 experiments performed. Culture of purified T and B cells in absence of 10 μg/ml GAM IgM served as control.

Table 3. Comparing various monomeric IgDs in blocking T-B interaction.[a]

Monomeric IgD	(μg/ml)	Inhibition of T - B interaction	Inhibition of Tδ - B interaction
KWD1	100	43%	30%
	10	39%	30%
	1	28%	29%
KWD9	100	87%	85%
	10	75%	62%
	1	37%	22%
KWD8	100	75%	77%
	10	64%	52%
	1	35%	27%
B1.8δ	100	34%	28%
	10	34%	21%
	1	33%	25%

[a] Results reflect blocking effects on T cell proliferative responses (detailed data are in figures 17-19).

figure 20, T cells were isolated from *in vivo* oligomeric IgD-treated or untreated (as control) heterozygous $IgD^{+/-}$ and homozygous $IgD^{-/-}$ mice, and stained with biotinylated IgD/avidin-FITC. FACS analysis revealed that T cells from both $IgD^{+/-}$ and $IgD^{-/-}$ mice have increased expression of IgD-R as compared with control T cells. Similar results were obtained with T cells from $IgD^{+/-}$ and $IgD^{-/-}$ mice when were treated with oligomeric IgD *in vitro*, as shown in figure 21. Therefore, whether treated *in vivo* or *in vitro* with oligomeric IgD, T cells from both $IgD^{+/-}$ and $IgD^{-/-}$ mice showed approximately 10% increased expression of IgD-R as compared with control T cells.

13. *Lack of immunoaugmenting effects of oligomeric IgD in $IgD^{-/-}$ mice: decreased antibody responses.*

Previous studies have demonstrated that oligomeric IgD failed to enhance antibody responses in athymic mice and $IgD^{-/-}$ mice (Coico et. al. 1988; Coico et. al. 1985; Swenson, 1995; Coico et. al. 1985). We have attributed the lack of immunoaugmenting activity of oligomeric IgD in athymic mice to the absence of T cells, and, hence Tδ cells, which mediate the functional activity of IgD. $IgD^{-/-}$ mice, on the other hand, fail to respond to oligomeric IgD *in vivo* despite the ability of T cells from such mice to upregulate IgD following IgD treatment in vivo and in vitro. Figures 22 and 23 illustrate the failure of oligomeric IgD to augment antibody responses to SRBC in IgD-deficient (IgD-knockout) mice. The spleen cells from control or IgD-treated mice were harvested 5 days following *in vivo* priming with SRBC. Anti-SRBC antibody responses were measured by ELISPOT assay. Spleen cells from $IgD^{-/-}$ showed fewer antibody forming cells regardless of whether IgD was injected together with antigen as compared with spleen cells from antigen-immunized, IgD-treated F1 ($IgD^{+/-}$) mice (figure 22). Moreover, spleen cells from

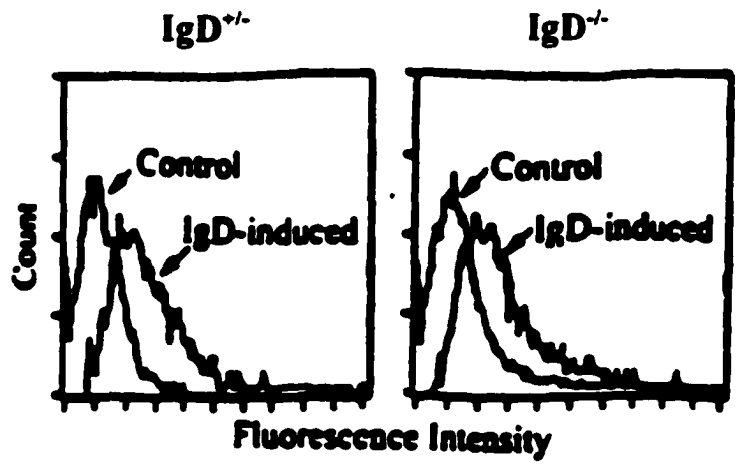

Figure 20. ***IgD-R can be upregulated on T cells from IgD-KO mice.*** Splenic T cells were isolated from untreated heterozygous IgD$^{+/-}$ and homozygous IgD$^{-/-}$ mice (control) or mice injected with 50μg oligomeric IgD on day -1 (IgD-induced). Cells were stained with 0.5μg of biotinylated-IgD/avidin-FITC and analyzed by flow cytometry. Results shown are representative of 5 experiments performed. Avidin-FITC yielded background have been subtracted.

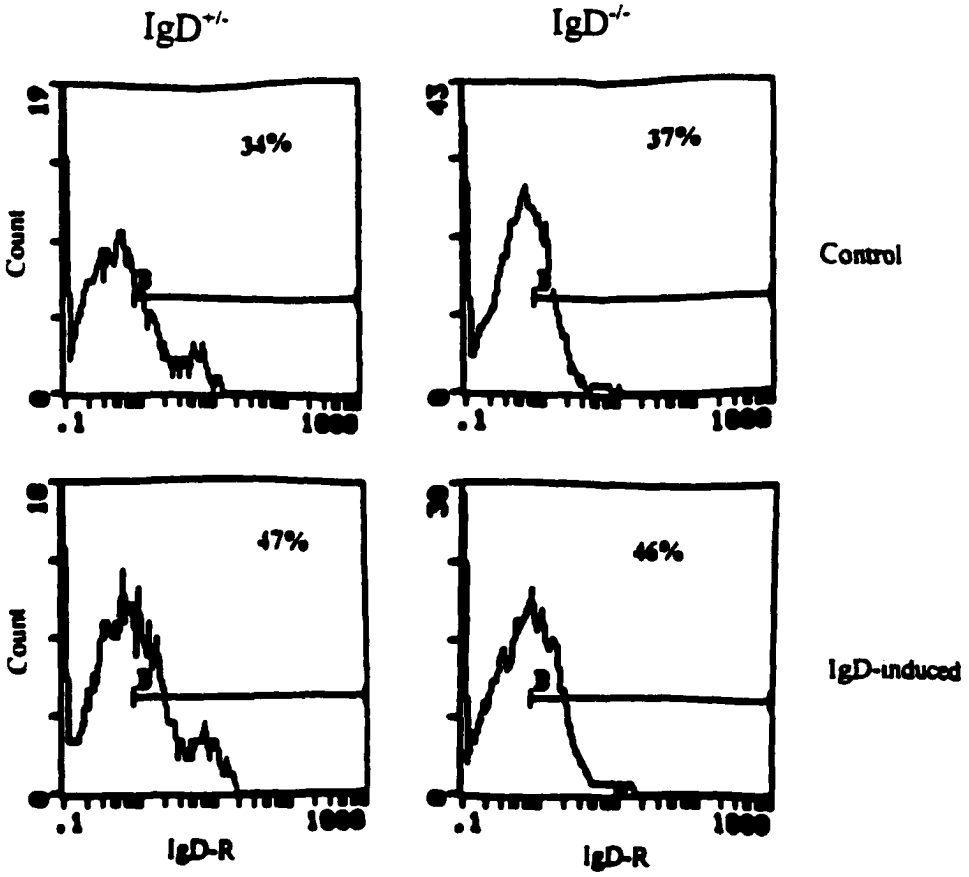

Figure 21. ***IgD-R can be upregulated on T cells from IgD-KO mice following in vitro treated with oligomeric IgD.*** Splenic T cells from heterozygous $IgD^{+/-}$ and homozygous $IgD^{-/-}$ mice were cultured on day -1 in absence the of oligomeric IgD (control) or in the presence of 50 μg/ml oligomeric IgD (IgD-induced). Cells were stained with 0.5 μg of biotinylated-IgD/avidin-FITC and analyzed by flow cytometry. Results shown are representative of 5 experiments performed (n=5). Avidin-FITC-yielded backgrounds have been subtracted.

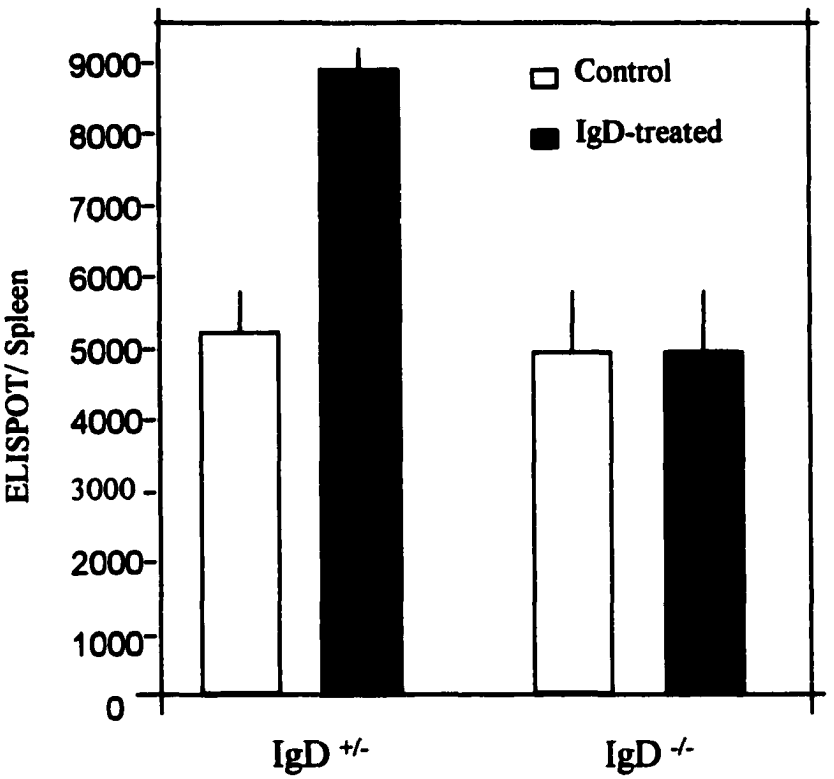

Figure 22. ***Lack of immunoaugmenting effects of oligomericIgD in IgD$^{-/-}$ mice as compared with IgD$^{+/-}$ mice.*** Heterozygous IgD$^{+/-}$ mice and homozygous IgD$^{-/-}$ mice were immunized i.v. with 1x10^{8} SRBC on day -5, with or without 100 μg of oligomeric IgD (n=5/group). Spleen cell ELISPOT assays were performed as described in Materials and Methods on day 0. BSA-coated membrane-yielded backgrounds have been subtracted. Resulted are expressed as the mean number of anti-SRBC ELISPOT per spleen.

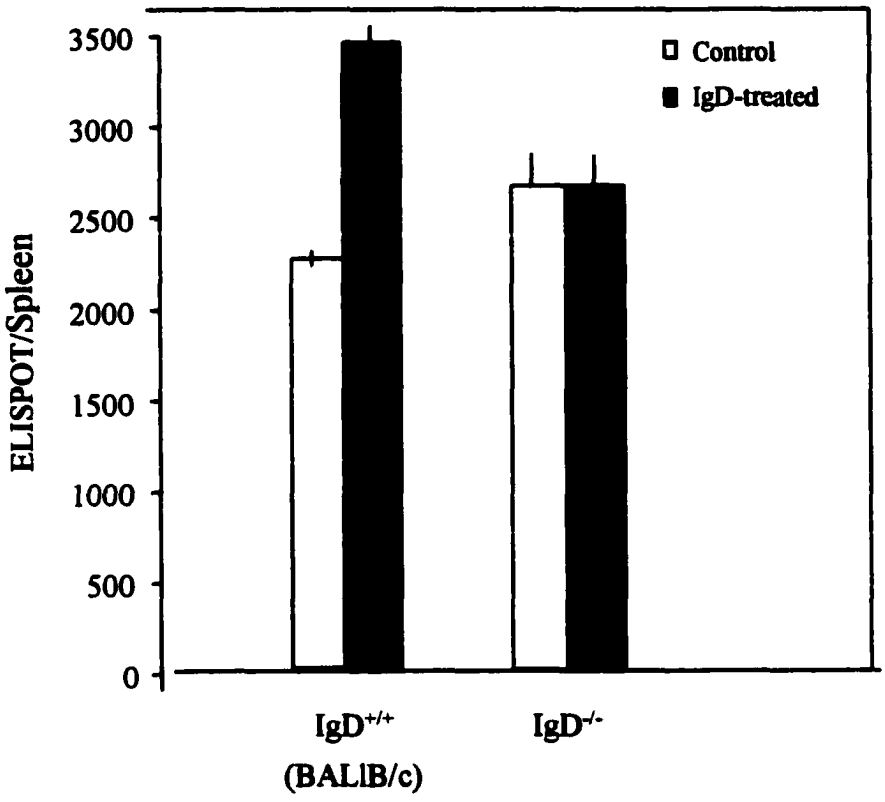

Figure 23. ***Lack of immunoaugmenting effects of oligomeric IgD in IgD$^{-/-}$ mice as compared with IgD$^{+/+}$ mice.*** IgD$^{+/+}$ BALB/c mice and homozygous IgD$^{-/-}$ mice were immunized i.v. with 1x10^{8} SRBC on day -5, with or without 100 μg of oligomeric IgD (n=5/group). ELISPOT assays were performed as described in Materials and Methods on day 0. BSA-coated membrane-yielded backgrounds have been subtracted. Resulted are expressed as the mean number of anti-SRBC ELISPOT per spleen.

IgD-treated BALB/c mice also gave increased antibody response when compared with spleen cells from IgD treated $IgD^{-/-}$ mice (figure23).

14. *T cells from $IgD^{+/+}$ but not $IgD^{-/-}$ mice show enhance proliferative response to antigen following in vivo treatment with oligomeric IgD.*

In light of the ability of T cells from $IgD^{-/-}$ mice to upregulate IgD-R in response to oligomeric IgD, we then asked whether in vivo induction of antigen-primed Tδ cells would predispose these cells to respond more vigorously to antigen *in vitro*. As demonstrated above, experiments using Ag-primed Tδ cells from BALB/c mice showed that upregulation of IgD-R on responding T cells facilitates their ability to respond to antigen when splenic B cells from normal mice (e.g. BALB/c) are used as APCs. In experiments described in this section, whole spleen cells from $IgD^{+/+}$ (BALB/c or C57B/6) versus $IgD^{-/-}$ mice were used to compare the responses of SRBC-primed cells from control or IgD-treated mice. The spleen cells were harvested 3 days following *in vivo* priming with SRBC. As shown in figures 24 and 25, spleen cells from homozygous IgD-knockout mice showed a similar level of proliferation regardless of whether they were previously injected with ologomeric IgD. However, spleen cells from IgD-treated homozygous $IgD^{+/+}$ mice gave significantly enhanced proliferative response in the presence of SRBC. In another experiment, whole spleen cells from $IgD^{+/-}$ versus $IgD^{-/-}$ mice were used to compare the responses of SRBC-primed cells from control or IgD-treated mice. We tested cells harvested 3 days following *in vivo* priming with SRBC, and anticipated that a portion of the antigen specific B cells in the heterozygous mice would still express membrane IgD at the time of cell harvest. As shown in figure 26, spleen cells from heterozygous $IgD^{+/-}$ (F1) mice, but not homozygous IgD-knockout ($IgD^{-/-}$) mice generated a two-fold higher poliferative response

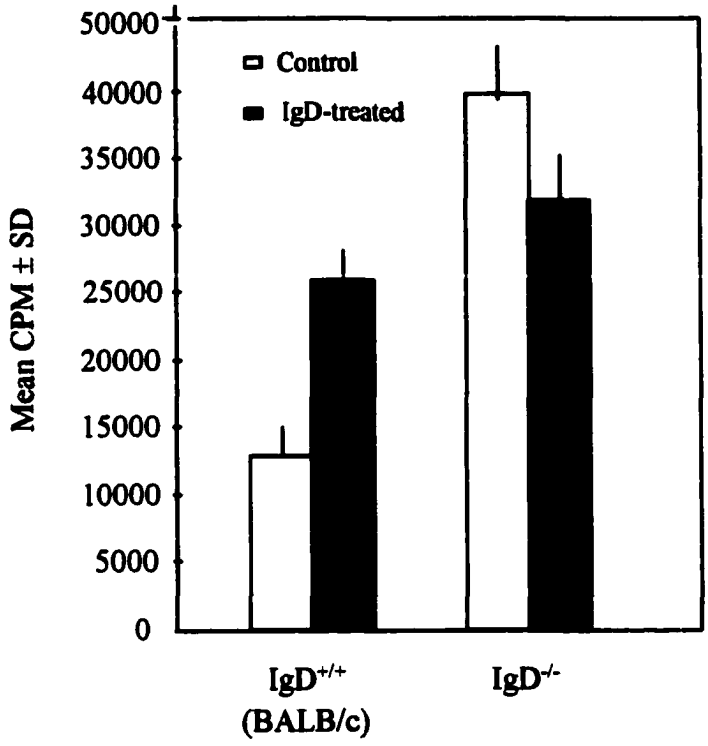

Figure 24. ***T cells from BALB/c (IgD$^{+/+}$) but not homozygous IgD$^{-/-}$ mice show enhanced responses to antigen following in vivo treatment with oligomeric IgD.*** BALB/c (IgD$^{+/+}$) and homozygous IgD$^{-/-}$ mice were immunized i.v. on day -3 with 1x10^{8} SRBC, with or without 100 μg of oligomeric IgD. In all experiments, IgD-R upregulation was confirmed by FACS analysis. In vitro cultures of spleen cells were established on day 0 at a density of 2x10^{5} cells/well in total volume of 0.2 ml with or without the presence of 20 μl of 0.1%SRBC. Following a 72 h culture period, cells were labeled with 1μCi ^{3}H-TdR for an additional 6 h, harvested and analyzed by scintillation counting. Results shown are representative of 5 experiments performed. Culture of non-IgD treated spleen cells in absence of SRBC served as a control.

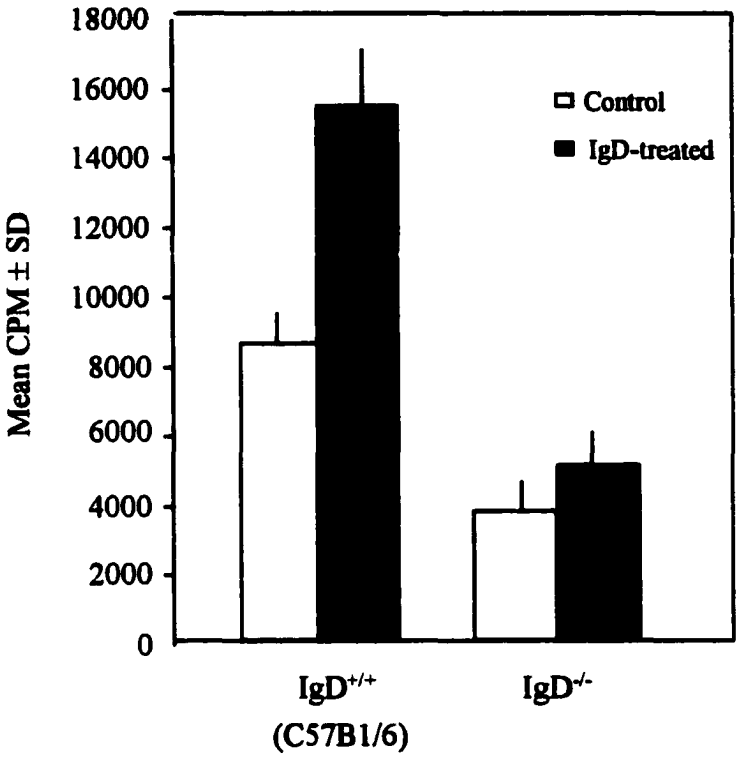

Figure 25. ***T cells from C57B1/6 ($IgD^{+/+}$) but not homozygous $IgD^{-/-}$ mice show enhanced responses to antigen following in vivo treatment with oligomeric IgD.*** C57B1/6 ($IgD^{+/+}$) and homozygous $IgD^{-/-}$ mice were immunized i.v. on day -3 with $1x10^8$ SRBC, with or without 100 μg of oligomeric IgD. In all experiments, IgD-R upregulation was confirmed by FACS analysis. In vitro cultures of spleen cells were established on day 0 at a density of $2x10^5$ cells/well in total volume of 0.2 ml with or without the presence of 20 μl of 0.1%SRBC. Following a 72 h culture period, cells were labeled with 1μCi ^{3}H-TdR for an additional 6 h, harvested and analyzed by scintillation counting. Results shown are representative of 5 experiments performed. Culture of non-IgD treated spleen cells in the absence of SRBC served as a control.

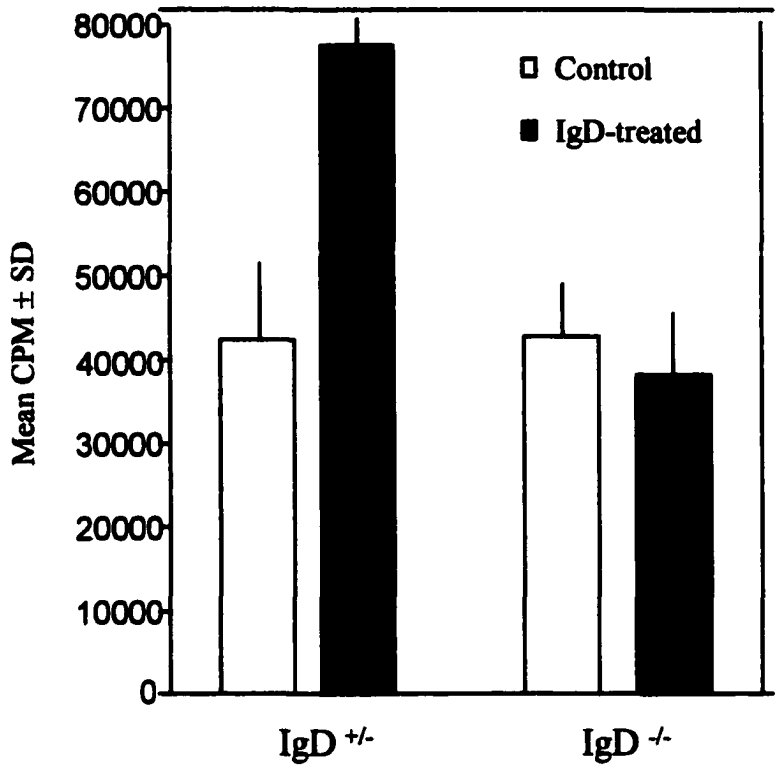

Figure 26. ***T cells from heterozygous IgD$^{+/-}$ but not homozygous IgD$^{-/-}$ mice show enhanced responses to antigen following in vivo treatment with oligomeric IgD.*** Heterozygous IgD$^{+/-}$ and homozygous IgD$^{-/-}$ mice were immunized i.v. on day -3 with 1x10^{8} SRBC, with or without 100 μg of oligomeric IgD. In all experiments, IgD-R upregulation was confirmed by FACS analysis. In vitro cultures of spleen cells were established on day 0 at a density of 2x10^{5} cells/well in total volume of 0.2 ml with or without the presence of 20 μl of 0.1%SRBC. Following a 72 h culture period, cells were labeled with 1μCi^{3}H-TdR for an additional 6 h, harvested and analyzed by scintillation counting. Results shown are representative of 5 experiments performed. Culture of non-IgD treated spleen cells in absence of SRBC served as a control.

when their T cells were induced to express IgD-R prior to antigen stimulation in vitro. Therefore, in agreement with our findings demonstrating the lack of IgD-induced enhancement of B cell responses to antigen, no enhancement of T cell responses to antigen was seen in $IgD^{-/-}$ mice despite upregulation of IgD-R by T cells.

15. *B cells from $IgD^{-/-}$ mice fail to present antigen to goat Ig-primed Tδ cells from $IgD^{+/-}$ mice as efficiently as B cells from $IgD^{+/-}$ mice.*

If interaction between IgD-R and IgD plays an important role in enhancing T cell and B cell responses, B cells from $IgD^{-/-}$ mice should fail to promote enhancement of responses to antigen by Tδ cells. To test this hypothesis, we prepared goat-Ig-primed T cells from $IgD^{+/-}$ mice, then induced IgD-R expression by injecting these antigen-primed mice with oligomeric IgD (50 μg) one day prior to harvesting splenic T cells. Splenic B cells were prepared from $IgD^{+/-}$ and $IgD^{-/-}$ mice and co-cultured with the goat-Ig-primed T and Tδ cells from $IgD^{+/-}$ mice together with GAM Ig as described in materials and methods. T cell proliferative responses were analyzed 72 hours later by ^{3}H-TdR incorporation. As shown in figure 27, responses of goat-Ig-primed Tδ cells were more than two-fold higher as compared with goat-Ig-primed control T cells only when B cells from the $IgD^{+/-}$ mice were used as APCs. Although B cells from the IgD-deficient mice were capable of serving as APCs to promote T cell responses to goat Ig, they lacked the capacity to elicit enhanced responses by the Ag-primed Tδ cells. These studies strongly suggest that expression of B cell membrane IgD is required to manifest enhanced responses by Tδ cells with upregulated IgD-R. Moreover, given the potent immunoregulatory properties of Tδ cells seen with regard to antibody responses, these results support the idea that T

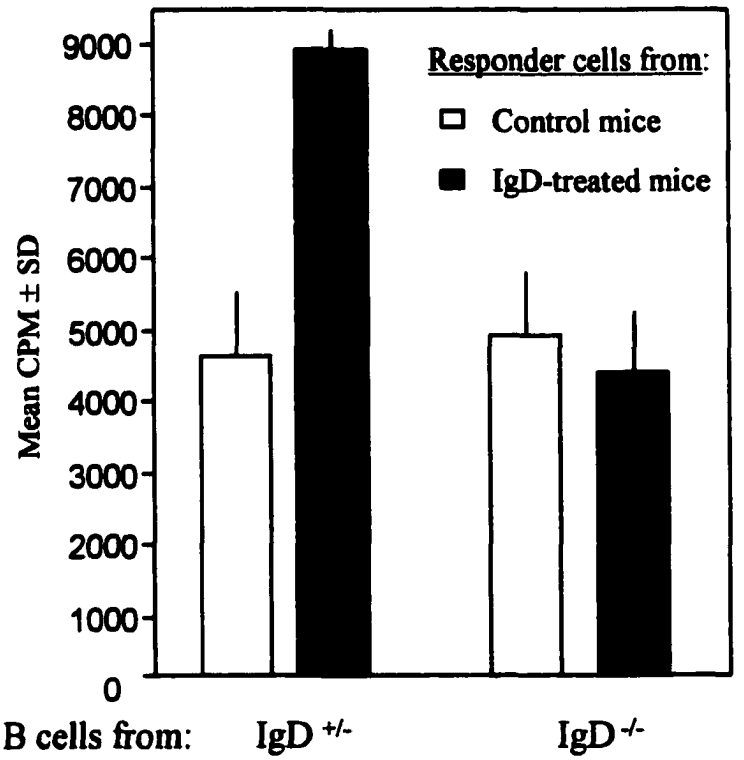

Figure 27. ***B cells from IgD$^{-/-}$ mice fail to present antigen to goat Ig-primed Tδ cells from IgD$^{+/-}$ mice as efficiently as B cells from IgD$^{+/-}$ mice.***
B cells were prepared form heterozygous IgD$^{+/-}$ mice and homozygous IgD$^{-/-}$ mice at the density of 1x10^5 cells /well, pulsed with 10 μg/ml GAM Ig and irradiated as described in Materials and Methods. Goat Ig-primed splenic T cells from IgD$^{+/-}$ mice treated with or without oligomeric IgD at the density of 1x10^5 cells/well were co-cultured with B cells for 72 h, then pulsed with 1μCi ^{3}H-TdR for and additional 6 h, harvested, and analyzed by scintillation counting. Results shown are representative of 5 experiments performed.

cell expression of IgD-R and B cell membrane IgD facilitate bidirectional signaling which occurs during T-B interaction (Figure E).

Part II: Investigation of co-stimulatory signaling events associated with upregulation of IgD-R.

1. Upregulation in vivo.

Activation of T cells requires two distinct signals: one signal is generated from the trimolecular interaction between TCR/CD3-CD4/CD8 and Ag-MHC, the second signal is dependent on interactions between key co-stimulatory molecules. B7/CD28 and CD40/CD40L pairs are critical co-stimulatory molecules involved in T-B cell activation. Previous studies in our lab demonstrated that interaction of IgD and IgD-R significantly enhances humoral immune responses (Coico et. al. 1984: Tamma et. al. 1991). We hypothesized that B cell membrane IgD and T cell membrane IgD-receptors may represent another ligand-receptor co-stimulatory pair that plays a role in T-B cell collaboration. In this study, we used immunofluorescence staining to study whether ligation of IgD-R causes transient expression of CD28 or CD40L on $CD4^+$ T cells, a phenotypic property of activated T cells. T cells were isolated from BALB/c mice that were treated one day earlier with oligomeric IgD. Expression of IgD-R, CD28 and CD40L were measured by immunofluorescence using 1) anti-CD4-PE and biotinylated-IgD/avidin-FITC; 2) anti-CD4-FITC and anti-CD28-PE; 3) anti-CD4-FITC and anti-CD40L-PE. Our data indicate that CD28 is transiently, indeed, upregulated on $CD4^+$ T cells following IgD-R cross-linking using oligomeric IgD (Figure 28). This finding provides evidence that interaction between IgD-R on $CD4^+$ T cells with IgD on B cells may play a role in the regulation of responses through CD28-mediated signaling. Expression of CD40L did not appear to be upregulated by this treatment as compared with cells from control mice.

Figure 28

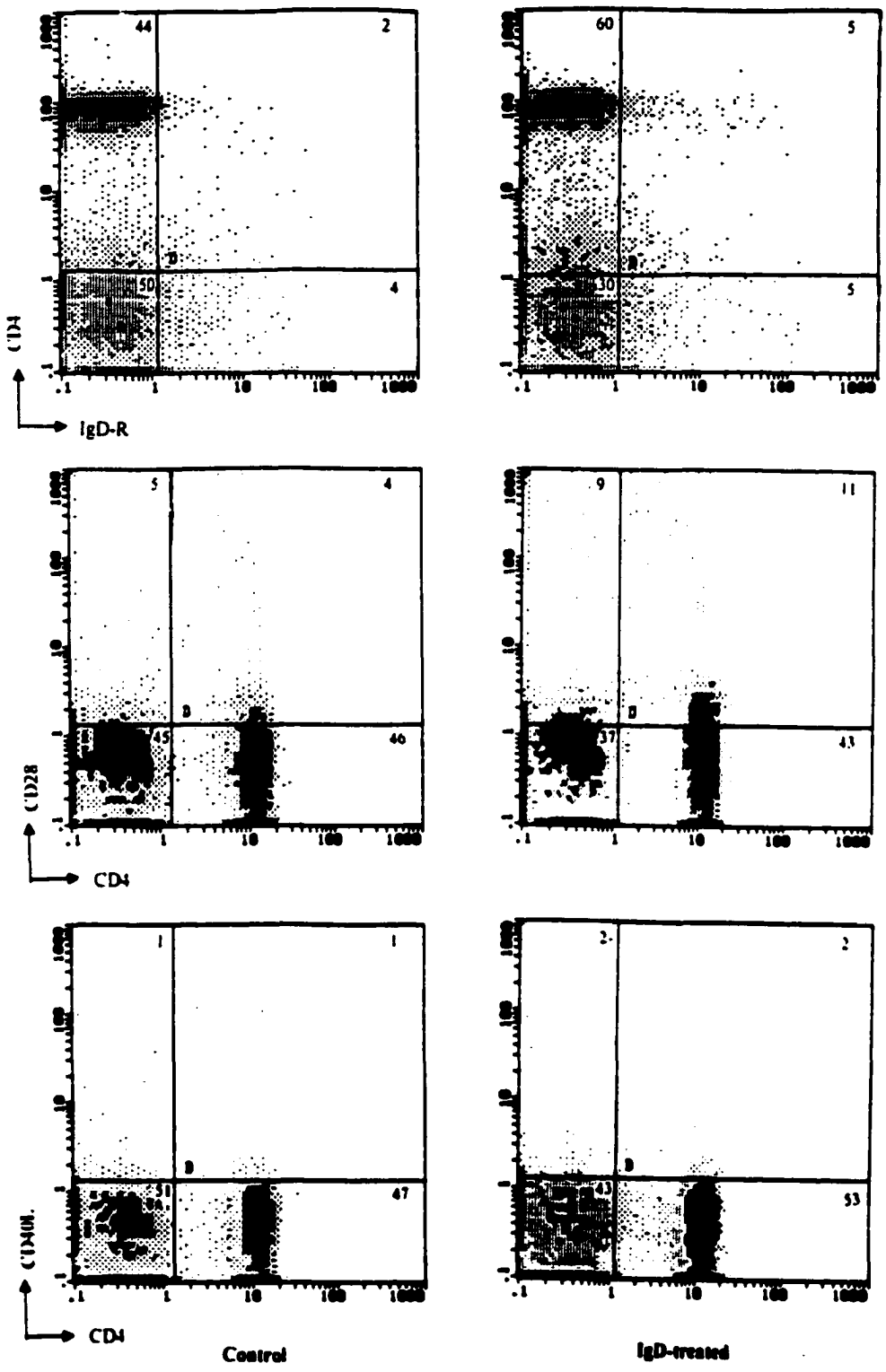

Figure 28. ***Increased expression of IgD-R and CD28 but not CD40L on T cells following in vivo treatment with oligomeric IgD.*** BALB/c mice were primed i.v. on day -1 with oligomeric IgD (100 μg) or with saline (as control). T cells were isolated and stained with anti-CD4-PE and biotinylated-IgD/avidin-FITC, anti-CD4-FITC and anti-CD28-PE or anti-CD40L-PE and analyzed by FACS. Results shown are representative of 3 experiments performed.

2. *Upregulation in vitro.*

We then asked that whether activation signals known to affect the expression of T cell costimulatory molecules also influence the expression of IgD-R. In these experiments, T cells were isolated from BALB/c mice then cultured for 4 h with control medium, anti-CD3 (1 μg/1 x 10^6 cells) or oligomeric IgD (10 μg/1 x 10^6 cells). Expression of IgD-R, CD28 and CD40L were measured by immunofluorescence using anti-CD4-PE and biotinylated-IgD/avidin-FITC, or anti-CD28-FITC, anti-CD40L-FITC, and results were compared to medium control. FACS analysis revealed that when T cells were treated with anti-CD3 or oligomeric IgD, the expression of IgD-R increased ~8-fold as compared with medium control cells (increased from 2% to 18%). The expression of CD28 also increased from 2% for control cells to 6% for treated cells (Figure 29). T cells stimulated with anti-CD3 or oligomeric IgD *in vitro* did not show any increased in expression of CD40L as compared to medium control. Analyses of T cell subsets for the expression of IgD-R, CD28 or CD40L simultaneously confirmed that these T cells were $CD4^+$. Taken together, these data indicate that *in vivo* and *in vitro* upregulation of IgD-R on T cells is associated with a transient upregulation of an important costimulatory molecule, CD28. Our failure to demonstrate upregulation of CD40L by immunofluorescence staining following T cell activation is not surprising in light of the fact that more sensitive assays (e.g. PCR) are often used to detect changes in the density of CD40L membrane expression.

Figure 29

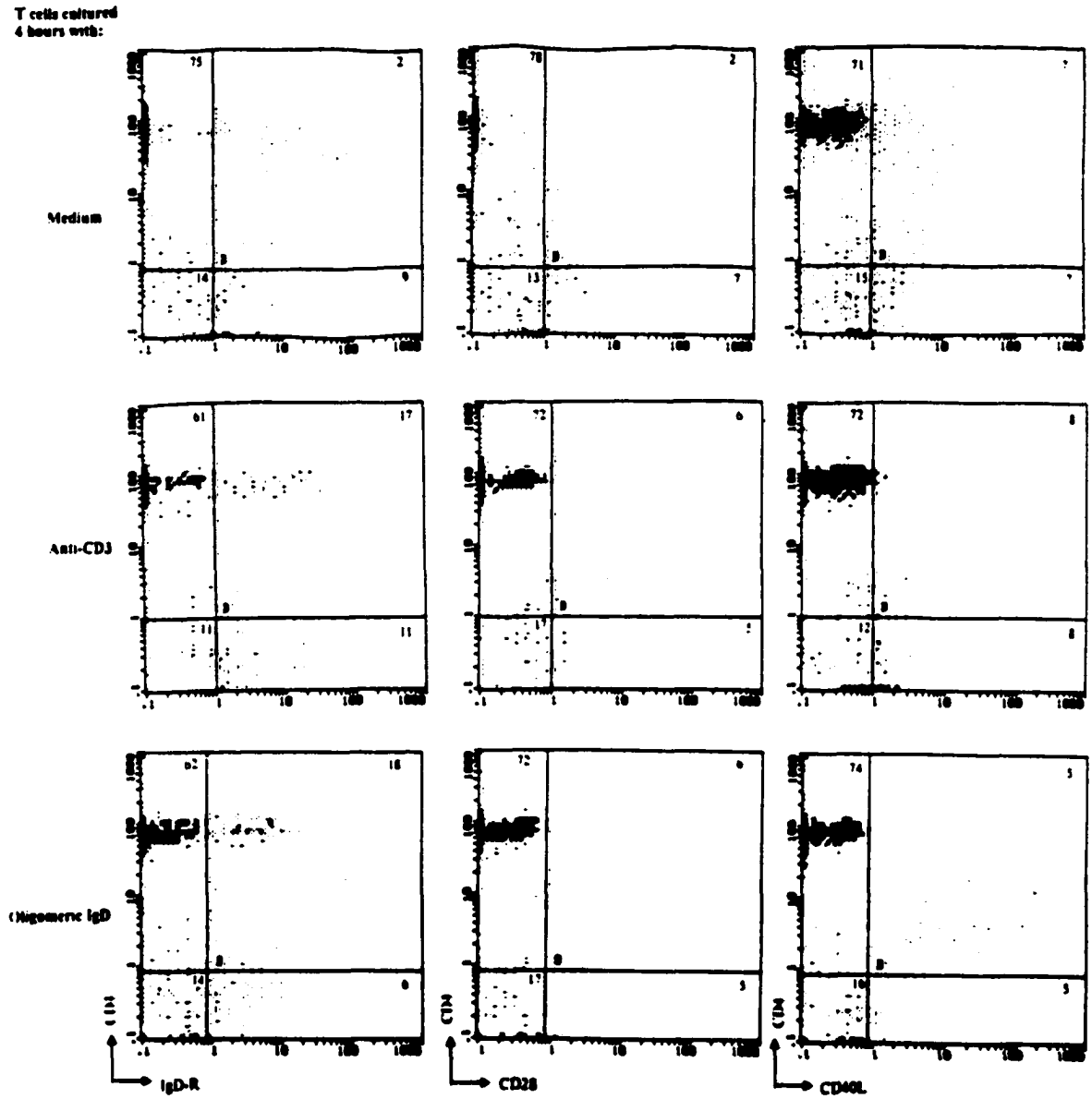

Figure 29. ***Increased expression of IgD-R and CD28 but not CD40L following in vitro treatment of T cells with anti-CD3 or oligomeric IgD.*** Splenic T cells from BALB/c mice were cultured for 4 h with anti-CD3 (1 μg/ml) or oligomeric IgD (10 μg/ml). After three washes, cells were stained with anti-CD4-PE and biotinylated-IgD/avidin-FITC, anti-CD28-FITC or anti-CD40L-FITC and analyzed by FACS. Results shown are representative of 3 experiments performed.

Part III: Study of the effects of upregulation of IgD-R on $CD4^+$ T cells with respect to cytokine production.

In vivo and *in vitro* studies have confirmed that exposure of $CD4^+$ T cells to oligomeric IgD results in upregulation of IgD-R on T cells and that this is correlated with significantly increased helper activities of these cells. Since the helper properties of T cells in the generation of antibody responses, are, in large part, mediated by the cytokines produced by $CD4^+$ cells, we examined whether exposure of T cells to oligomeric IgD leads to increased cytokine production. Previous studies using ELISAs have suggested that cytokine levels are increased in the supernatants of T cell cultures following exposure of such cells to oligomeric IgD (Coico et al. 1990). Since cytokine ELISAs are relatively insensitive and because we were interested in analyzing the T_H1 versus T_H2 cytokine profile of T cells with upregulated IgD-R, we used two approaches to analyze this question. First, intracellular cytokine staining was used to analyze production of IL-2, INFγ, IL-4 and IL-5 following *in vivo* and *in vitro* treatment with oligomeric IgD. We also , examined whether there was any relationship between increased IgD-R upregulation and cytokine production. In the second approach, we employed a ribonuclease protection assay to simultaneously assess production of IL-2, INFγ, IL-4 and IL-10 mRNA levels in $CD4^+$ T cells following *in vivo* and/or *in vitro* treatment with oligomeric IgD. Our data, summarized below, demonstrates that exposure of T cells to oligomeric IgD results in induction of IL-4, IL-5, IL-10 mRNA synthesis. Based on cytokine analyzes, it appears that interaction between IgD-R on $CD4^+$ T cells and IgD on B cells may lead to activation of T_H2 subset T lymphocytes.

1. ***Intracellular cytokine staining: Induction of IL-4, IL-5 synthesis by in vivo treatment with oligomeric IgD.***

T cells were isolated from the spleens of BALB/c mice primed with 50 μg of oligomeric IgD on day -3 and -1, and intracellular cytokine profiles were measured using 1) anti-CD4-PE and anti-INFγ-FITC or anti-IL2-FITC, and 2) anti-CD4-FITC and anti-IL-4-PE or anti-IL-5-PE. As shown in figure 30A, compared with control T cells, FACS analysis revealed an increased synthesis of IL-4, IL-5 by IgD-R$^+$, CD4$^+$ T cells. The percentage of IL-4-producing cells increased from 12% in control CD4$^+$ T cells to 28% in CD4$^+$ T cells primed *in vivo* with oligomeric IgD. Similarly, the percentage of cells producing intracellular IL-5 increased from 10% in control CD4$^+$ T cells to 25% in CD4$^+$ T cells primed with oligomeric IgD *in vivo*. While the percentage of cells synthesizing of IL-2 and INFγ was found to be marginal, as shown in figure 30A, the percentage of cells producing intracellular IL-2 increased from 21% in control CD4$^+$ T cells to 24% in CD4$^+$ T cells primed in vivo with oligomeric IgD. Similarly, a marginal increase in the frequency of INFγ-producing cells from 8% (control) to 11% (IgD-treated) was observed. Furthermore, examination of cells producing IL-4 and IL-5 associated with IgD-R expression using biotinylated-IgD/avidin-FITC vs anti-IL-4-PE or anti-IL-5-PE, showed that both IgD-R expression and IL-4 synthesis were simultaneously upregulated as shown in figure 30B.

2. ***Treatment with oligomeric IgD in vitro induced synthesis of IL-4 and IL-5 by CD4$^+$ T cells.***

Figure 30A

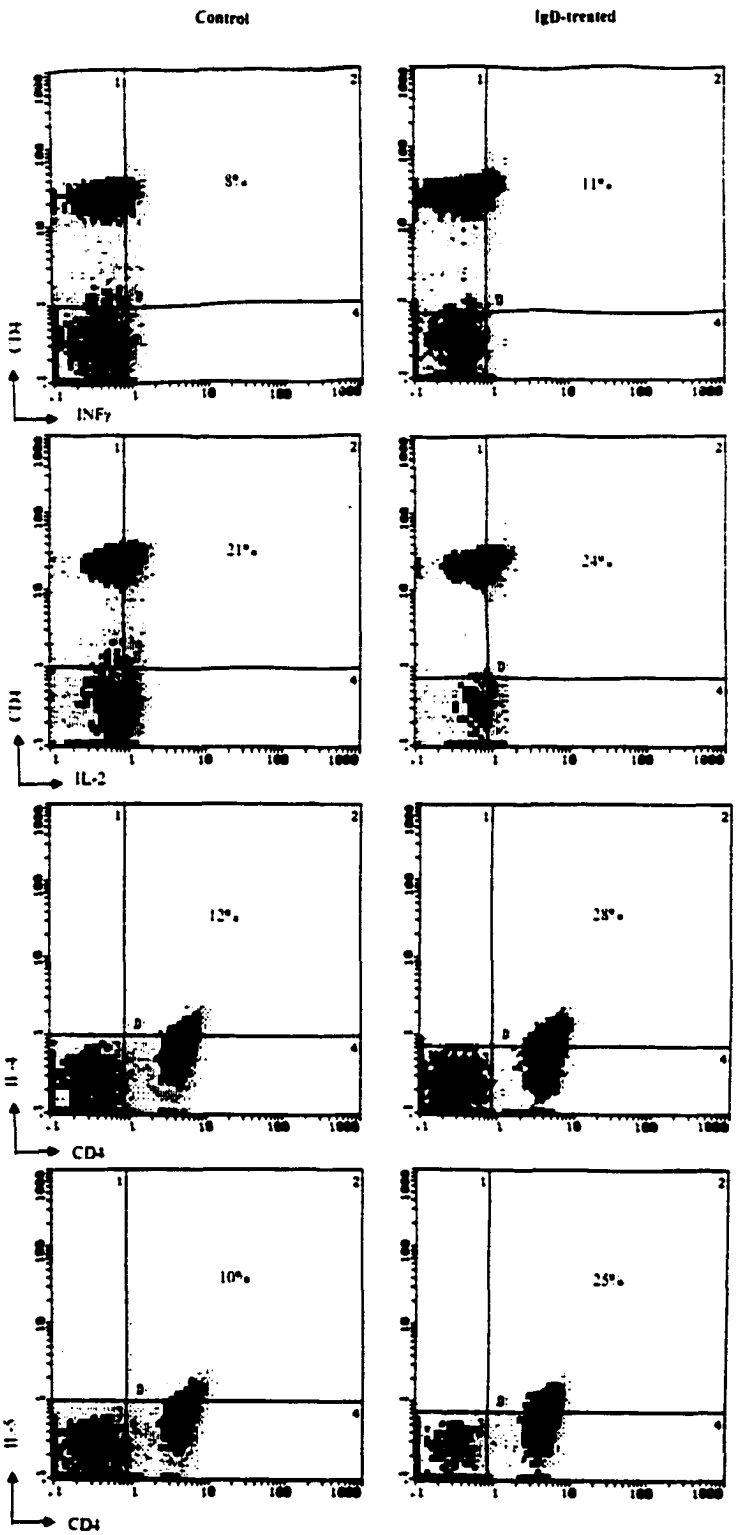

Figure 30B

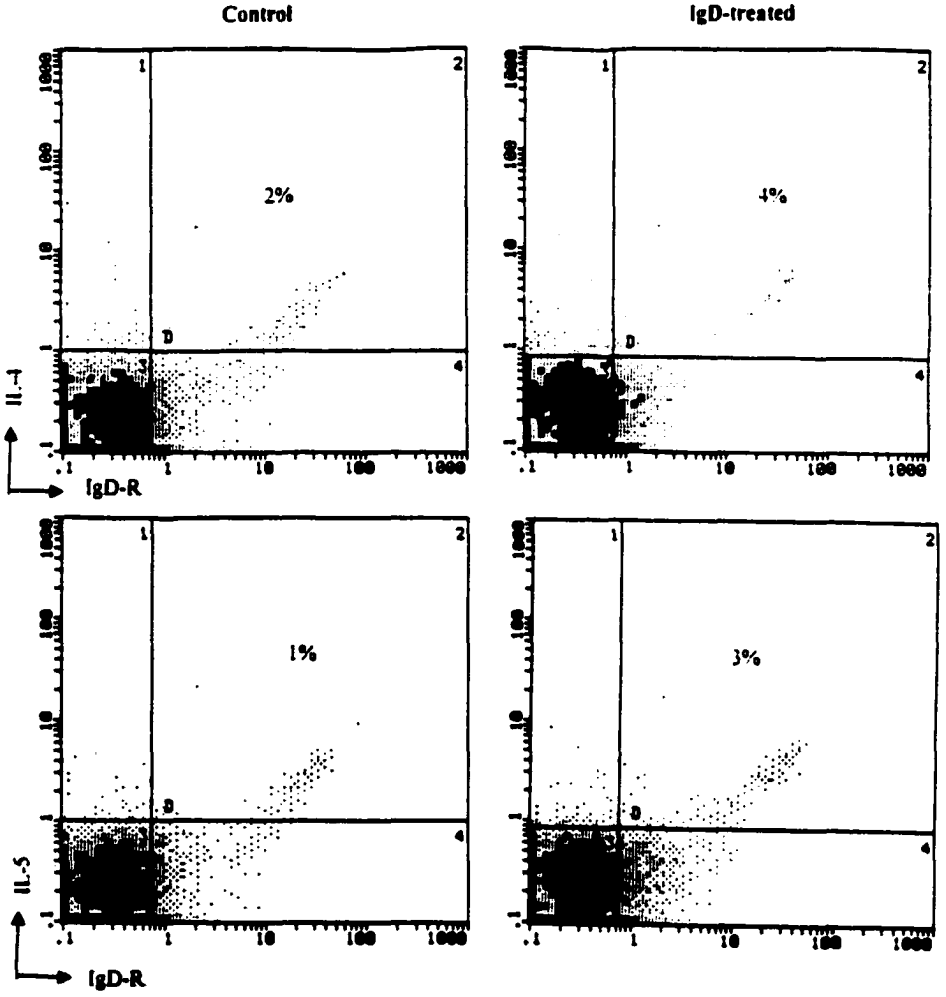

Figure 30. ***Increased production of cytokines IL-4 and IL-5 following in vivo treatment with oligomeric IgD.*** BALB/c were primed i.v. on day -3, -1 with oligomeric IgD (100 μg) or with saline (as control). (A) T cells were isolated and stained with anti-CD4-FITC and anti-IL-4-PE or anti-IL-5-PE; anti-CD4-PE and anti-IL-2-FITC or anti-INFγ-FITC. (B) T cells were stained with biotinylated-IgD/avidin-FITC and anti-IL-4-PE or anti-IL5-PE. Cells were then analyzed by FACS. Results shown are representative of 4 experiments performed.

In these experiments, T cells from BALB/c mice were cultured for 4 h with control medium or in the presence of 10 μg/ml of oligomeric IgD. Intracellular cytokine synthesis was measured as described above. FACS analysis revealed result similar to those cells from mice treated with oligomeric IgD. As shown in figure 31A, the percentage cells producing intracellular IL-4 following IgD treatment increased approximately 10% as compared to $CD4^+$ T cells cultured in medium alone. The percentage of cells making intracellular IL-5 increased by ~9% as compared to $CD4^+$ T cells cultured in medium alone. There were no significant changes in the percentage of cells producing intracellular IL-2 and INFγ by $CD4^+$ T cells treated with oligomeric IgD as compared to $CD4^+$ T cells cultured in medium alone.

Next, we examined IL-4 and IL-5 synthesis associated with IgD-R expression by utilizing biotinylated-IgD/avidin-FITC vs anti-IL-4-PE or anti-IL-5-PE. The data indicate that both IgD-R expression and IL-4 and IL-5 synthesis were simultaneously upregulated, as shown in figure 31B. These studies suggested that the upregulation of IgD-R on $CD4^+$ induces the synthesis of cytokines consistent with the T_H2 phenotype -- a phenotype known to be associated with B cell helper activity.

3. *Induction of cytokine gene transcription by treatment with oligomeric IgD in vivo and in vitro.*

We then analyzed the cytokine mRNA profiles of T cells following *in vivo* or *in vitro* treatment with oligomeric IgD. In these experiments, T cells isolated from control BALB/c mice were treated *in vitro* for 4 h with: 1) medium; 2) oligomeric IgD (10 μg/10^6 cells); 3) PMA (100 ng/10^6 cells) + ionomycin (400 ng/10^6 cells). Total cellular RNA

Figure 31A

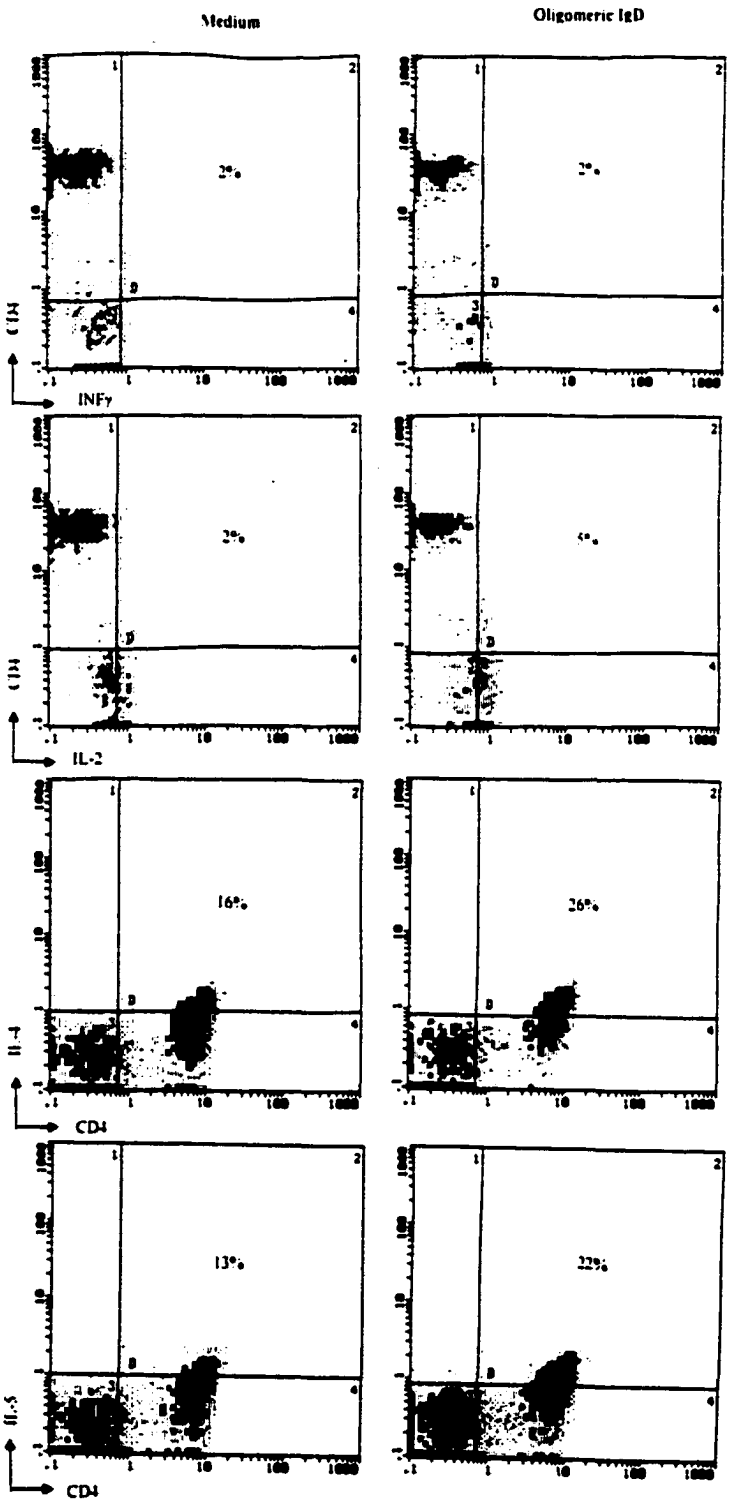

Figure 31B

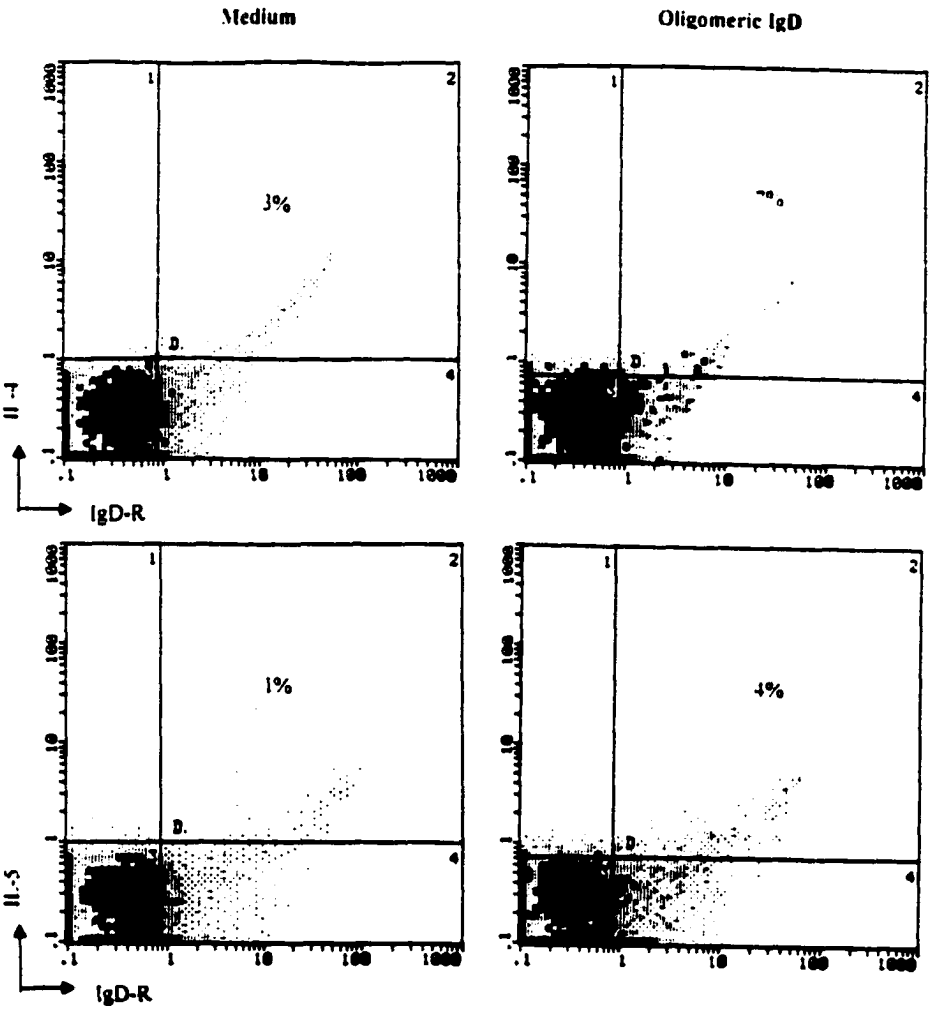

Figure 31. ***Increased production of cytokines IL-4 and IL-5 following in vitro Treatment of T cells with oligomeric IgD.*** Splenic T cells from BALB/c mice were cultured for 4 h with oligomeric IgD (10 μg/ml). (A) After three washes, T cells were stained with anti-CD4-FITC and anti-IL-4-PE or anti-IL-5-PE; anti-CD4-PE and anti-IL-2-FITC or anti-INFγ-FITC. (B) T cells were stained with biotinylated-IgD/avidin-FITC and anti-IL-4-PE or anti-IL-5-PE. Cells were then analyzed by FACS. Results shown are representative of 4 experiments performed.

from 5 x 10^6 cells was extracted and used for ribonuclease protection assays (details of this assay are provided in the materials and methods section). As shown in figure 32, compared to RNA from T cells treated with medium alone, treatment with PMA + ionomycin (positive control sample) induced IL-2, INFγ, IL-4 and IL-10 mRNA expression. Treatment with oligomeric IgD induced IL-4 and IL-10 mRNA expression without affecting IL-2 or INFγ mRNA expression.

RNA was then extracted from T cells harvested from spleens of BALB/c mice exposed *in vivo* to oligomeric IgD to analyze cytokine gene transcription. We also performed experiments in which T cells were exposed *in vivo* to oligomeric IgD followed by subsequent *in vitro* exposure to oligomeric IgD. As shown in figure 32, compared to RNA from control T cells (harvested from untreated mice), treatment with oligomeric IgD *in vivo* plus *in vitro* further increased specific IL-4, IL-10 mRNA without affecting IL-2 and INFγ mRNA expression. Treatment with oligomeric IgD *in vivo* alone did not induce IL-2, INFγ, IL-4 mRNA in T cells but enhanced IL-10 mRNA expression as compared to RNA from control T cells. We also analyzed the data by normalizing against L32 or GAPDH gene transcription (L32 and GAPDH are housekeeping genes). As shown in figures 33 and 34, when analyzed in this way, the data clearly indicate that treatment of T cells with oligomeric IgD induced IL-4 and IL-10 cytokine gene transcription, supporting the intracellular cytokine staining results described above.

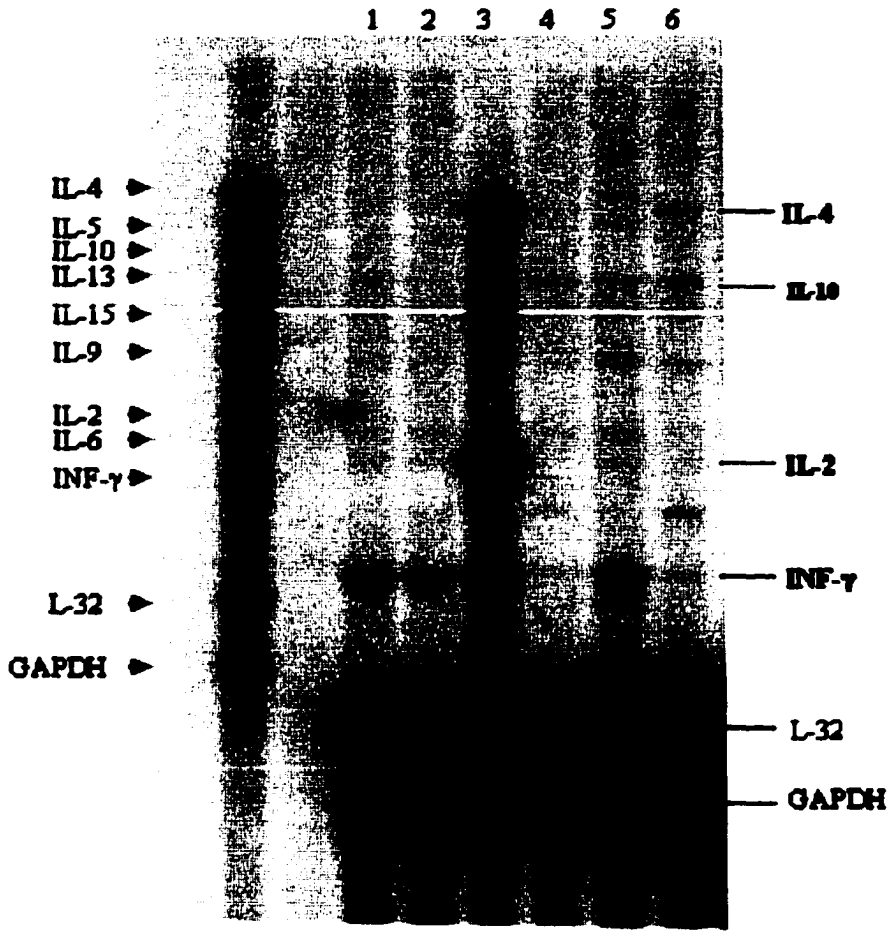

Figure 32. ***Induction of cytokine gene transcription following treatment with oligomeric IgD in vivo and in vitro.*** Purified T cells from BALB/C mice were stimulated, and total RNA from 5 x 10^6 T cells was extracted. Cytokine mRNA levels were analyzed by RNase protection assay as described in materials and methods. Lane 1: control; lane 2: medium; lane 3: PMA (100 ng/10^6 cells) + ionomycin (400 ng/10^6 cells); lane 4: oligomeric IgD *in vitro* (10 μg/10^6 cells); lane 5: oligomeric IgD *in vivo* (50 μg); lane 6: oligomeric IgD *in vivo* (50 μg) and *in vitro* (10 μg/10^6 cells).

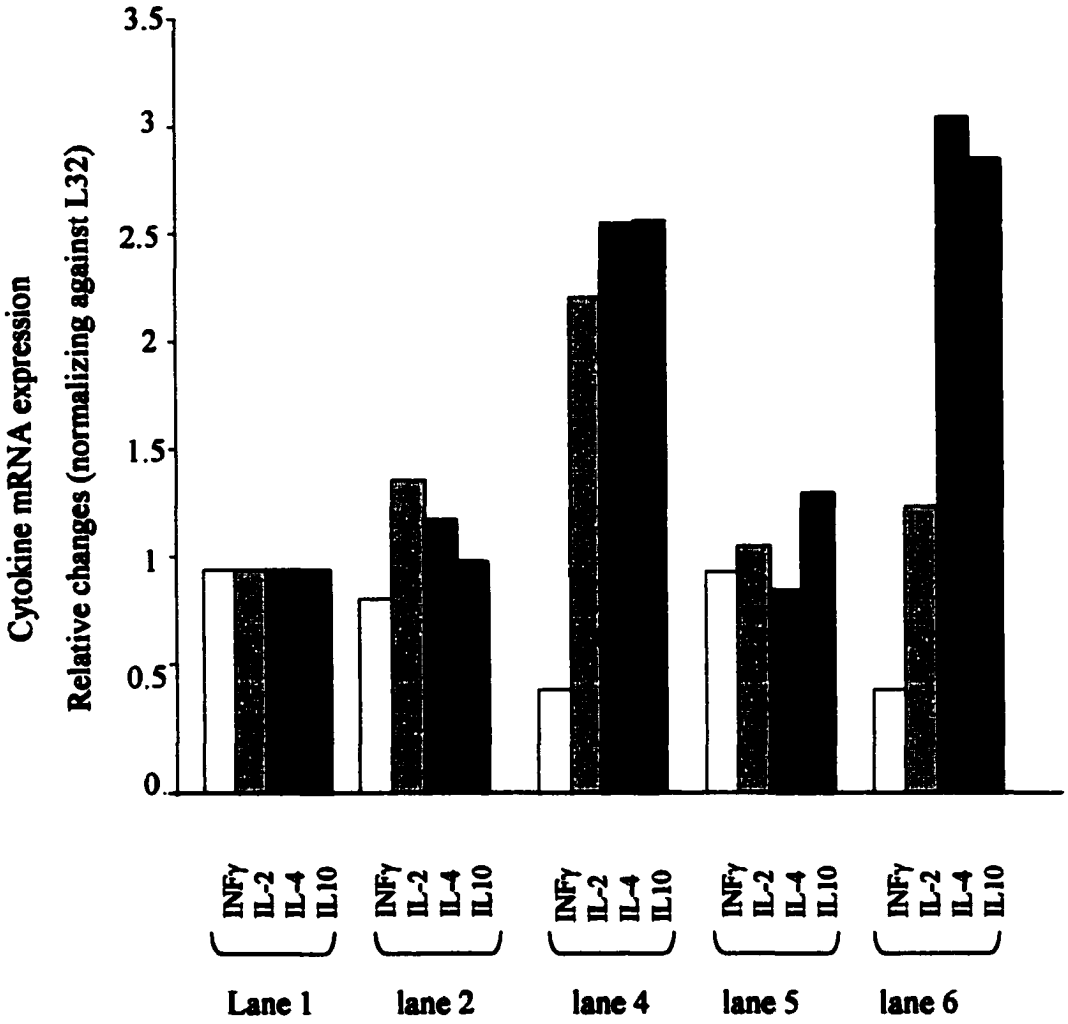

Figure 33. ***Induction of cytokine gene transcription following treatment with oligomeric IgD in vivo and in vitro.*** Lane 1: RNA from control T Cells; lane 2: RNA from medium treated T cells; lane 4: RNA from in vitro oligomeric IgD treated T cells; lane 5: RNA from in vivo oligomeric IgD treated T cells; lane 6: RNA from in vivo and in vitro oligomeric IgD treated T cells.

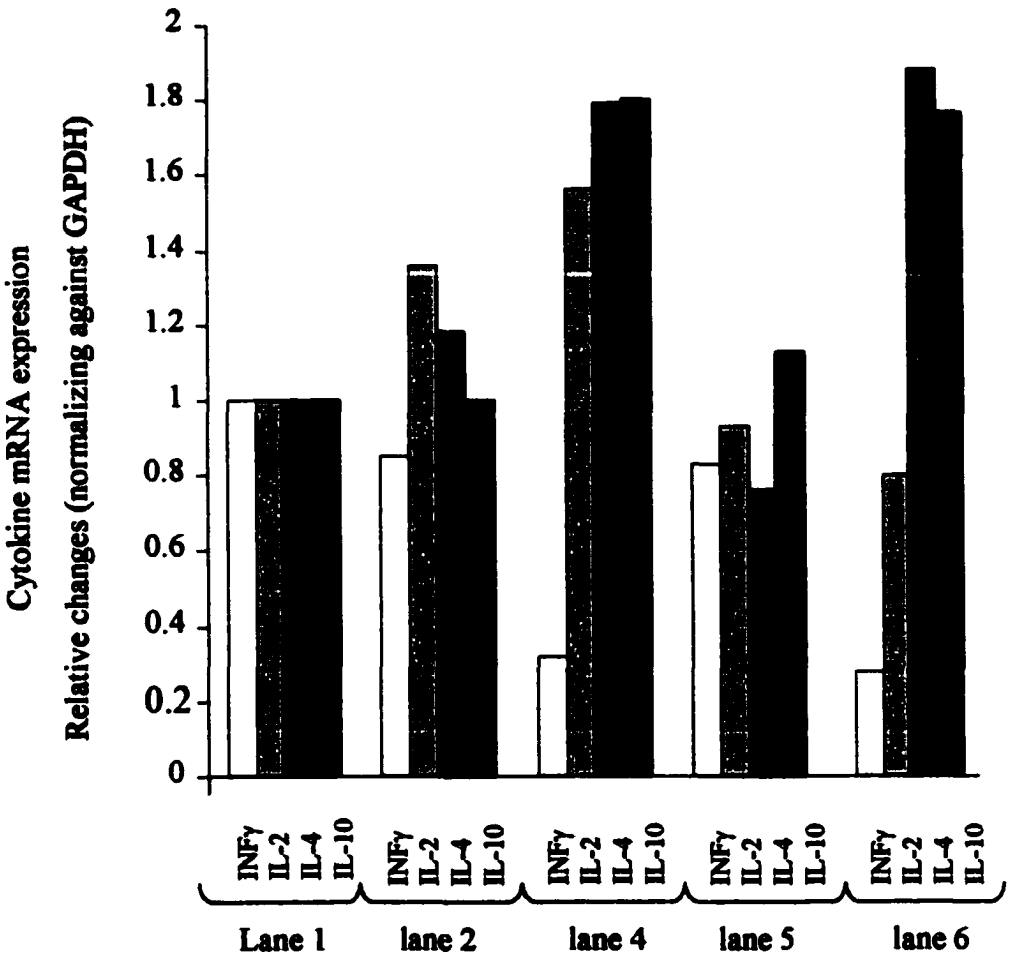

Figure 34. ***Induction of cytokine gene transcription following treatment with oligomeric IgD in vivo and in vitro.*** **Lane 1: RNA from control T cells; lane 2: RNA from medium treated T cells; lane 4: RNA from in vitro oligomeric IgD treated T cells; lane 5: RNA from in vivo oligomeric IgD treated T cells; lane 6: RNA from in vivo and in vitro oligomeric IgD treated T cells**

Part IV. IgD-R mediated signal transduction

Taken together, results summarized in parts 1-3 of this thesis suggest that upregulation of IgD-R on T cells facilitates cognate T-B interactions by mediating bidirectional signaling. This conclusion is based upon our findings that interactions between B cells from normal ($IgD^{+/+}$ or $IgD^{+/-}$) but not IgD knockout ($IgD^{-/-}$) mice interact with T cells with upregulated IgD-R result in: (a) increased antibody responses, and (b) clonal expansion of antigen specific T cells together with cytokine production consistent with a T_H2 profile. It is reasonable to expect that such effects on B and T cells are mediated by signal transduction events in both populations. We examined signal transduction in T hybridoma cells known to constitutively express IgD-R to determine whether cross-linking of IgD-R leads to activation of one or more protein tyrosine kinase (PTK) activation pathways. Murine T hybridoma cells, 7C5 constitutively express IgD-R as has been confirmed by staining. In addition, IgD-R expression can be upregulated in 7C5 cells following stimulation with PMA + ionomycin or with oligomeric IgD.

Earlier studies have shown that inhibitors of protein tyrosine kinase (PTK) completely prevented upregulation of IgD-R in response to oligomeric IgD suggesting that cross-linking of IgD-R may induce signal transduction and functional consequences through one or more PTK activation pathways leading to upregulation of IgD-R (Swenson et. al. 1993; Amin et. al. 1993). In results summarized below, we show that cross-linking of IgD-R on 7C5 cells by oligomeric IgD results in: 1) signal transduction as evidenced by tyrosine phosphorylation of several intracellular proteins; 2) the appearance of a ~29 kDa band which is phosphorylated and exhibits strong affinity for biotinylated IgD (putative IgD-R membrane protein); and, 3) tyrosine phosphorylation of the putative IgD-R itself as shown by

immunoprecipitation and immunoblotting experiments.

We further examined the effect of IgD-R cross-linking on the expression of Fas and Fas-L, the molecules that have been shown to be associated with apoptosis induction. Cells positive for both Fas and Fas-L increased dramatically following overnight culture even though slightly lower than that in cells treated with dexamethasone. Further, prior cross-linking of IgD-R and sebsequence treatment with dexamethasone gave similar percentages of double-positive cells as in cells that had IgD cross-linked. IgD cross-linking-induced apoptosis remained similar to that in cells cultured in medium alone. Pretreatment in which IgD-R cross-linking occurred was associated with lower Fas/Fas-L expression as compared with treatment with dexamethasone alone. These data indicate prior ligation with oligomeric IgD (i.e., IgD-R cross-linking) may provide protection against apoptosis induction. Thus ligation of IgD-R may predispose antigen-specific T lymphocytes for survival during primary responses when IgD^+ B cellls serve as antigen-presenting cells.

Finally, we established that 7C5 cells also constitutively express the T cell activation marker CD69. The function of CD69 is not yet established although it is well known that it is a very early T cell activation marker which is transiently expressed on normal T cells following antigen activation and mediates signal transduction following cross-linking. Our interest in examining CD69 was to determine whether this marker might itself be the IgD-R

1. *IgD-R are constitutively expressed on 7C5 T hybridoma cells.*

7C5 T hybridoma cells constitutively express much higher levels of IgD-R than resting splenic T cells (Swenson et. al. 1993). IgD-R expression was measured by FACS using biotinylated-IgD. As shown in figure 35A, 70% of 7C5 cells bind to biotinylated-

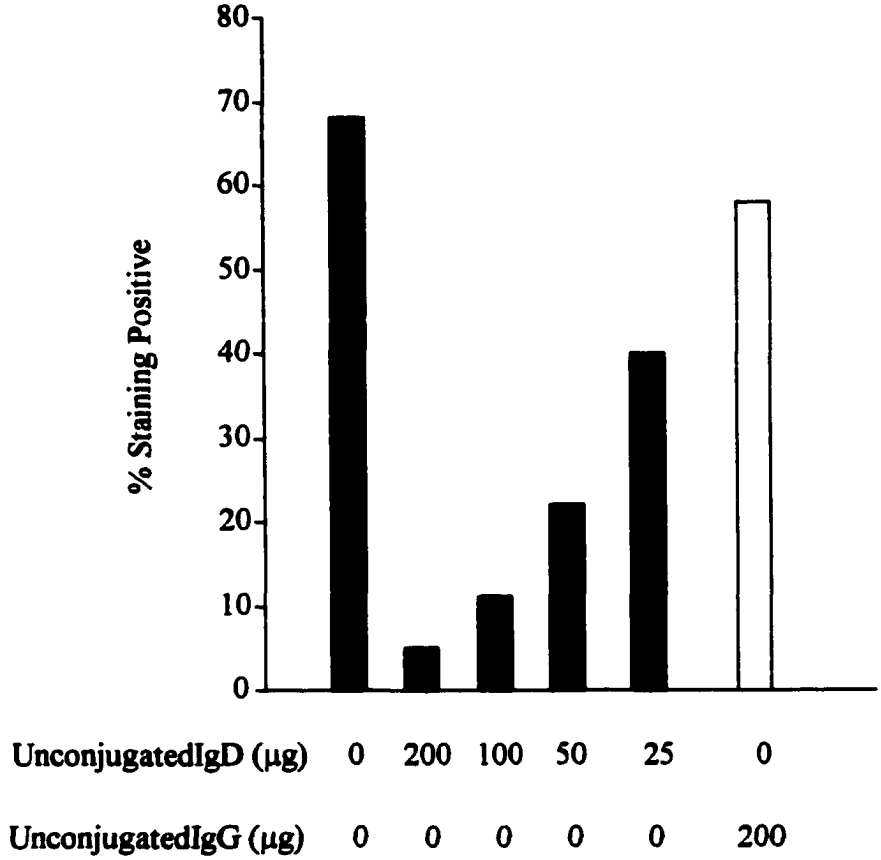

Figure 35A. ***Competitive inhibition of IgD-R on 7C5 T hybridoma cells.*** 7C5 T hybridoma cells were stained with biotinylated IgD (0.5 μg /10^6 cells) /avidin-FITC in the presence or absence of unconjugated IgD or IgG at the doses indicated. Samples were analyzed by FACS. Results shown are representative of 2 experiments performed. Avidin-FITC-yeilded backgrounds have been subtracted.

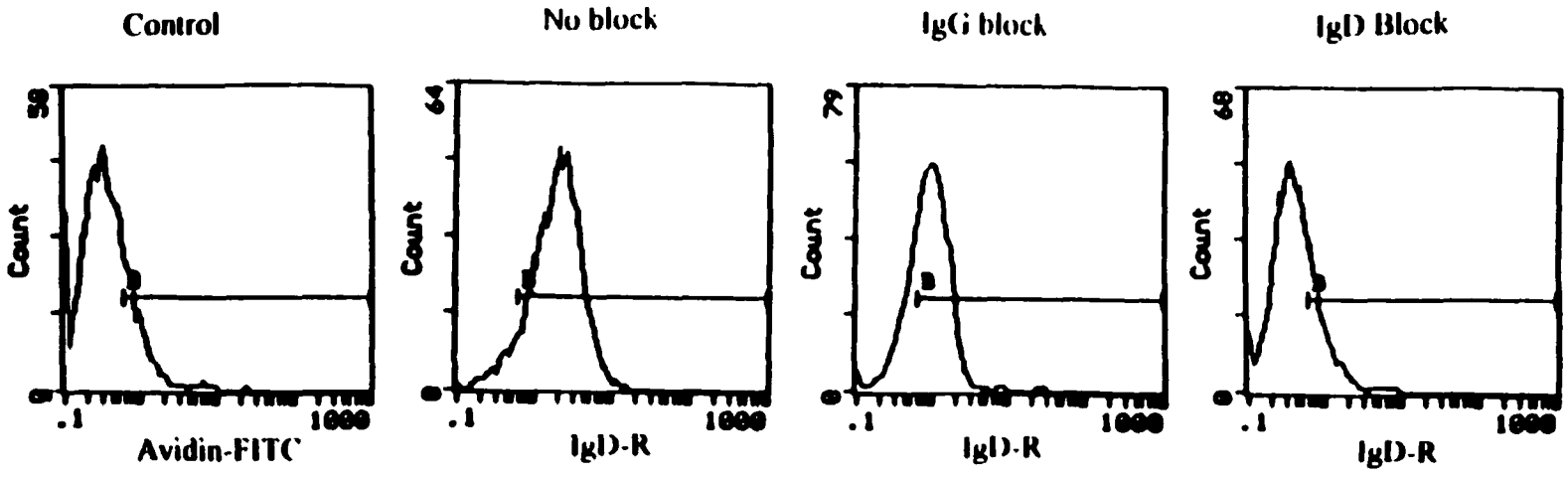

Figure 35B. ***Murine T hybridoma cells, 7C5 constitutively express IgD-R.*** 7C5 cells were analyzed by FACS during log phase of growth by staining with 0.5 μg of biotinylated IgD followed by avidin-FITC. Specificity of IgD-R binding to IgD is confirmed by blocking with unconjugated mouse IgG (100 μg) or mouse IgD (100 μg). Cells stained with avidin-FITC served as control.

IgD. In other experiments, the percentage of 7C5 cells which are IgD-R$^+$ varied (ranging from 35%-70%) depending on the number of 7C5 cell passages from cryopreserved stocks. This is not a phenomenon unique to 7C5 T hybridoma cells and the membrane receptor under investigation. Therefore, as a general practice, when the level of IgD-R expression was found to decrease below 35% following continued passage of these cells, we have routinely discarded the cells being grown and used a newly thawed cryopreserved batch for subsequent experiments.

When 7C5 cells were incubated with unconjugated IgD prior to staining with biotinylated-IgD, binding of biotinylated-IgD was blocked in a dose-dependent manner (Figure 35A). The IgD specificity of these IgD-R was further confirmed by showing that only IgD, but not IgG, blocked immunofluorescence staining with biotinylated-IgD (Figure 35B).

2. *IgD-R can be upregulated on 7C5 T hybridoma cells.*

We observed that IgD-R expressed on 7C5 cells can also be upregulated either by treatment with oligomeric IgD or with PMA + ionomycin, as measured by biotinylated-IgD/avidin-FITC staining (figure 36). These studies demonstrated that the T hybridoma cell line, 7C5, constitutively expresses IgD-R, and furthermore, that this cell line can be utilized to further characterize the biological properties of IgD-R (e.g. intracellular signaling pathways following IgD-R cross-linking) and, possibly, the structural and molecular features of this receptor.

3. *Analysis of CD69 expression on 7C5 T hybridoma cells.*

IgD-R have been shown to be rapidly upregulated following exposure to different stimuli, including antigenic stimulation, anti-CD3, PMA + ionomycin, IgD-containing

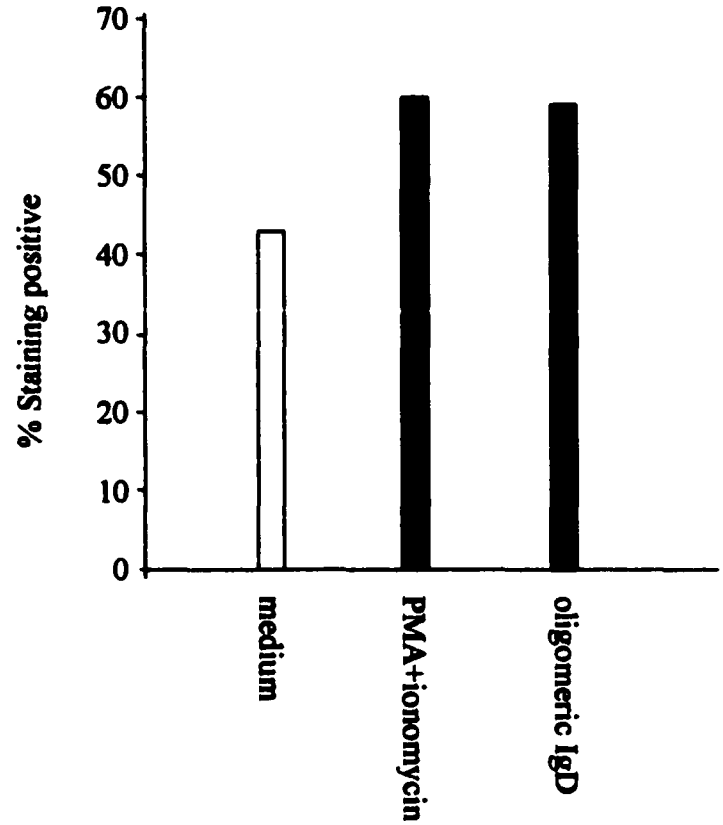

Figure 36. ***IgD-R can be upregulated on 7C5 T hybridoma cells.*** 7C5 T hybridoma cells were stimulated for 60 minutes with medium, PMA (100 ng/10^6 cells) + ionomycin (1μM) and oligomeric IgD (10 μg/10^6 cells). After three washes, cells were stained with biotinylated IgD (0.5 μg/10^6 cells)/avidin -FITC and analyzed by a FACS. Results shown are representative of 3 experiments performed. Avidin-FITC-yeilded backgrounds have been subtracted.

immune complex, cytokine IL-2 and IL-4. (Coico et. al. 1985b; Coico et. al. 1985c; Coico et. al. 1988; Coico et. al. 1988) As noted above, given the kinetics of the well-known T cell activation marker, CD69, whose function has not yet been resolved, we examined whether this marker might, in fact, be the IgD-R which is also transiently expressed following T cell activation. In the initial studies, we analyzed by immunofluorescence whether IgD-R$^+$, 7C5 T hybridoma cells expressed CD69. As shown in figure 37, when 7C5 cells were stained with biotinylated anti-CD69/avidin-FITC and analyzed by FACS, we found that ~40% of the cells constitutively express CD69. We then examined whether unconjugated IgD inhibited the binding of biotinylated anti-CD69 to these cells. 7C5 cells were incubated with various amounts of unconjugated IgD as shown in figure 37 prior to staining with biotinylated anti-CD69. The data indicate that binding of biotinylated anti-CD69 to 7C5 cells was not blocked by unconjugated IgD with ~40-43% of the cells remaining CD69$^+$ (Figure 37). These results indicate that CD69 and IgD-R are two distinctly different molecules and that CD69 appears to exhibit no specificity for IgD.

4. *Increased expression of IgD-R following in vitro treatment of 7C5 T hybridoma cells with anti-CD69.*

Since it was clear that CD69 is expressed on 7C5 cells and it is known that antibody specific for CD69 is capable of inducing T cell proliferation (Cebrian, et.al. 1988), it was of interest to determine whether cross-linking of CD69 can also cause upregulation of IgD-R. The rationale for this experiment is, again, based upon the idea that T cells express a redundant array of co-stimulatory molecules which, upon ligation, regulate other costimulatory molecules following T cell activation. 7C5 cells were cultured for 30 minutes

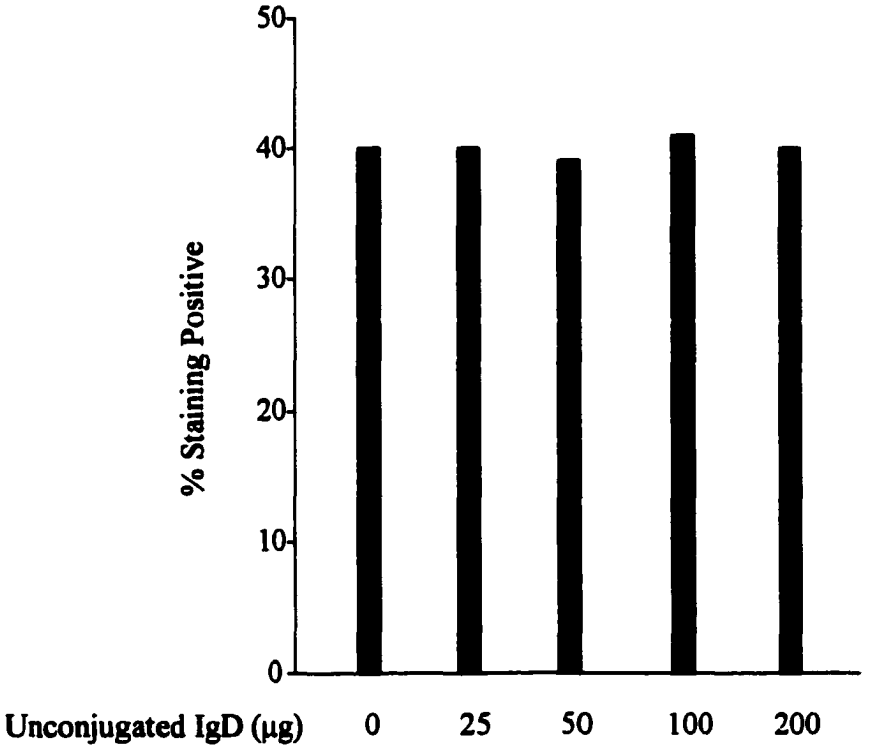

Figure 37. ***Detection of CD69 on 7C5 T hybridoma cells.*** 7C5 T hybridoma cells were stained with biotinylated anti-CD69 (1 μg/10^6 cells)/Avidin-FITC in the presence or absence of increasing concentration of unconjugated IgD. Samples were analyzed by FACS. Results shown are representative of 2 experiments performed. Avidin-FITC-yielded backgrounds have been subtracted.

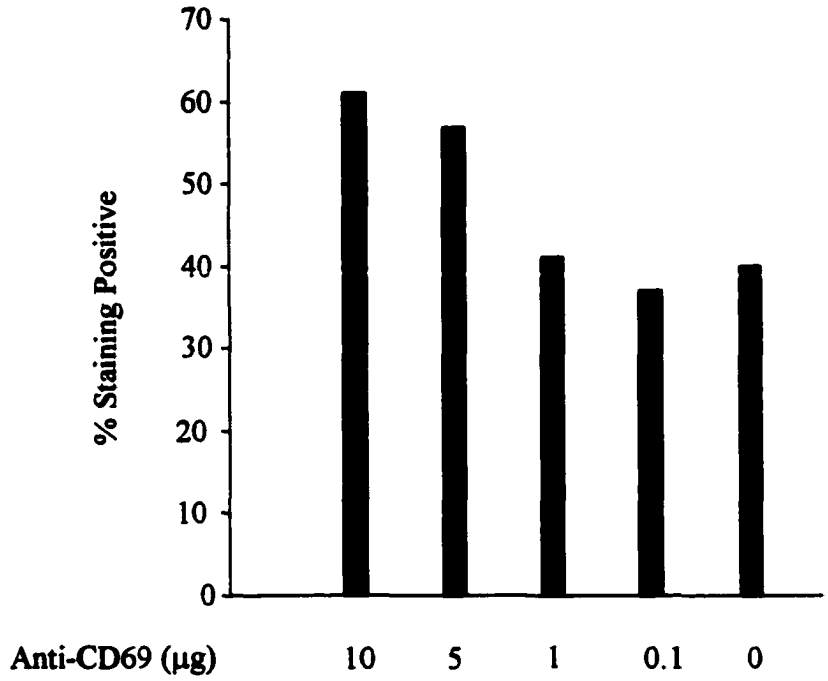

Figure 38. ***Expression of IgD-R following in vitro treatment of 7C5 T hybridoma cells with anti-CD69.*** 7C5 T hybridoma cells were cultured for 30 minutes with anti-CD69 at the doses indicated. After three washes, cells were stained with biotinylated-IgD (0.5 μg/10^6 cells)/avidin-FITC and analyzed by FCAS. Results shown are representative of 3 experiments performed. Avidin-FITC-yielded backgrounds have been subtracted

with anti-CD69 at 10μg, 5μg, 1μg, 0.1μg, respectively. Expression of IgD-R was then analyzed by FACS analysis using biotinylated IgD and avidin-FITC. As shown in figure38, treatment of 7C5 cells with anti-CD69 was also able to increase IgD-R expression from 38% to 61% in the experiment shown. These results indicate that like the signal transduction pathway(s) mediated by cross-linking of CD3, ligation of CD69 appears to coordinately upregulate IgD-R expression.

5. *IgD-R crosslinking induces tyrosine phosphorylation of intracellular proteins in 7C5 cells.*

We then performed experiments to directly assess the ability of 7C5 cells to respond with evidence of signal transduction following ligation of IgD-R. T hybridoma cells were stimulated by cross-linking IgD-R using oligomeric IgD. As positive controls, 7C5 cells were also stimulated with PMA + ionomycin, and by CD3 cross-linking (Tamma et. al., 1997). Cell lysates were precleared and immunoprecipitated with anti-phosphotyrosine antibody coupled to protein G + A-agarose beads. Following extensive washing, immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting with anti-phophotyrosine antibody. As shown in figure 39, there are additional proteins being tyrosine phosphorylated, and the intensity of the banding pattern is greater in IgD-R cross-linked samples when compared to samples from medium control. As noted above, stimulation with PMA + ionmycin and with CD3-cross-linking were used as positive controls. These studies indicate that IgD-R cross-linking induces tyrosine phosphorylation of several intracellular proteins. Pre-ligation of IgD-R followed by CD3 ligation also results in enhanced phosphorylation of intracellular proteins.

We then performed experiments in which 7C5 cells were stimulated with PMA + ionomycin, cell lysates were precleared as described in materials and methods, and then immunoprecipitated with anti-phosphotyrosine antibody coupled to protein G plus A-agarose beads, followed by immunoblotting with biotinylated-IgD/streptavidin. The purpose of this experiment was to determine whether T cell activation signals induce the expression of an IgD-binding protein. As shown in figure 40, two bands at ~29 and ~34 kDa were visible. In another experiment, cell lysates were immunoprecipitated with IgD-Sepharose after preclearing, and then immunoblotted with anti-phosphotyrosine antibody and biotinylated-IgD/streptavidin, respectively. As shown in figure 41 and 42, two tyrosine phosphorylated bands at ~29 and 34 kDa were consistently seen. We speculated that one of both of these proteins at ~29 and 34 kDa exhibit specificity for IgD.

We next examined whether these bands were specifically binding or non-specifically binding to IgD. Cells were stimulated with T cell activation stimuli (PMA + ionomycin, etc.) as described in materials and methods. Cell lysates were precleared, immunoprecipitated with anti-phosphotyrosine antibody coupled to protein G + A-agarose, and blots were incubated with unconjugated oligomeric IgD prior to incubation with biotinylated IgD followed by streptavidin treatment. Only the lower band at ~29 kDa was blocked by unconjugated IgD (figure 43). Therefore, it appears that the upper 34kda band binds to biotinylated IgD non-specifically.

Cell lysates were then immunoprecipitated with IgD-sepharose after preclearing, and, then probed with anti-Ig-HRP-conjugated antibody to test whether immunoprecipitated lysates were contaminated by Ig light chain. Results showed that the 34 kDa band appears to be a contaiminting Ig light chain, as shown in figure 44. Taken together, these experiments

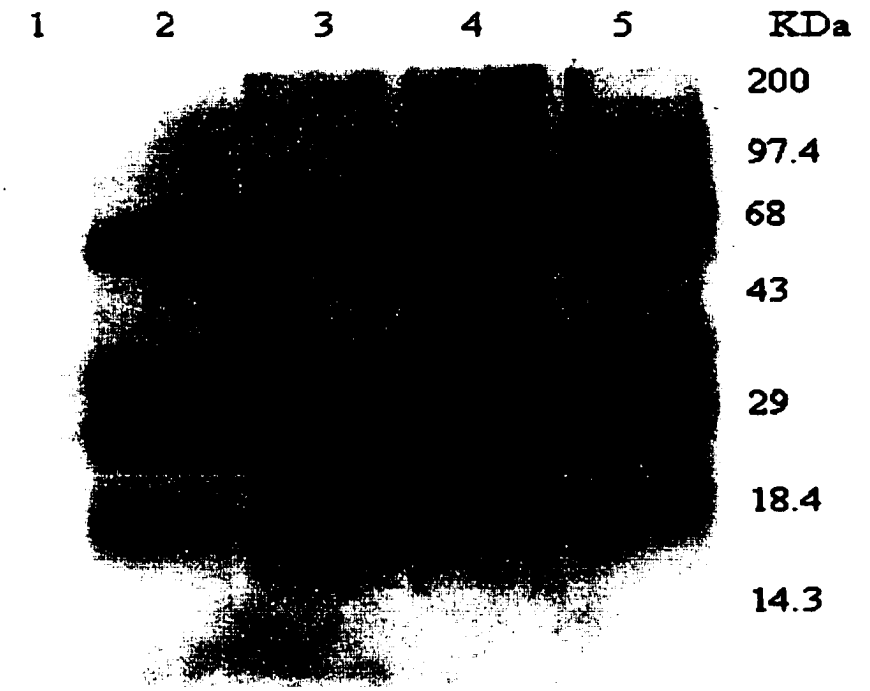

Figure 39. ***IgD-R cross-linking induces tyrosine phosphorlation of intracellular Proteins.*** **7C5 cells were stimulated, lysed and pre-cleared; lysates were immunoprecipitated with phosphotyrosine antibody coupled to protein G + A-agarose beads, and immunobloted with anti-phosphotyrosine antibody. Blots were developed by using the ECL system according to the manufacturer's instructions. Lane 1: Mol. Wt. Std.; lane 2: medium Control; lane 3: PMA + ionomycin; lane 4: CD3 cross-linking; and lane 5: IgD-R cross-linking.**

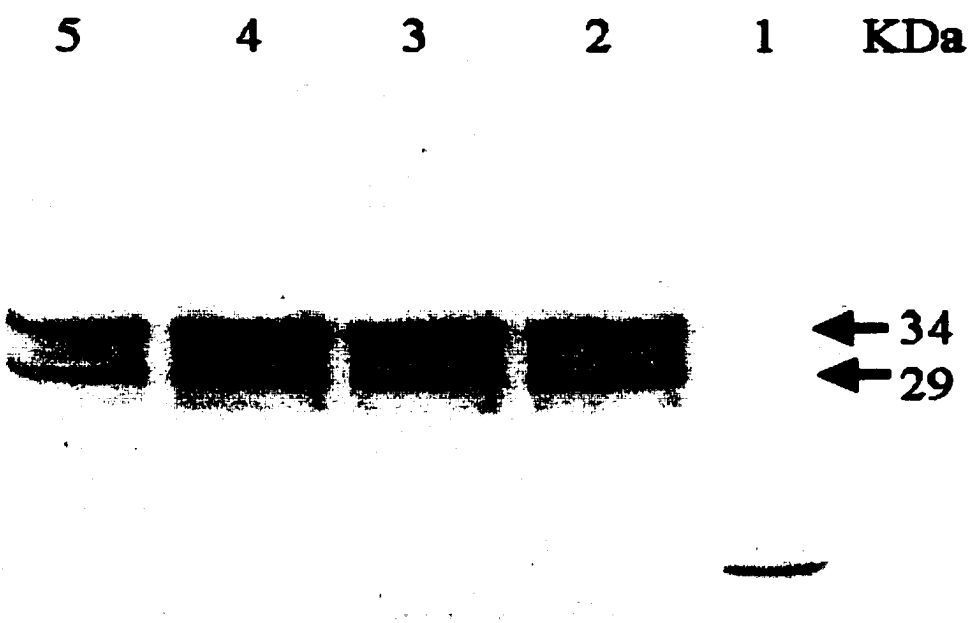

Figure 40. ***Tyrosine phosphorylation of IgD-specific protein.*** 7C5 cells were stimulated and lysed. Cell lysates were pre-cleared followed by immunoprecipitation with IgD-Sepharose beads as described in materials and methods, and analyzed by immunoblotting with anti-phosphotyrosine antibody. Lane 1: Mol. Wt. Std.; lane 2: PMA + ionomycin; lane 3: CD3 cross-linking; lane 4: IgD-R cross-linking followed by CD3 cross-linking; lane 5: IgD-R cross-linking.

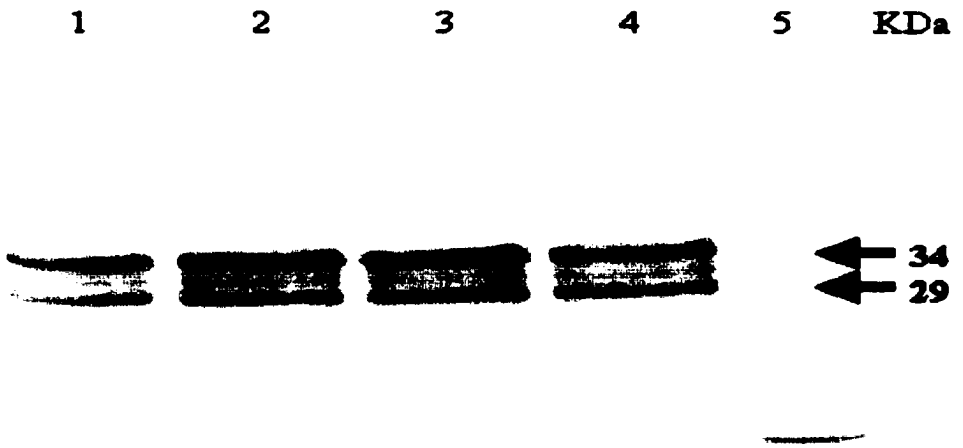

Figure 41. ***Phosphorylation of IgD-R.*** **7C5 cells were stimulated and lysed. Cell lysates were pre-cleared, immunoprecipitated with anti-phosphotyrosine antibody coupled to protein G + A-agarose beads. Immunoprecipitates were analyzed by immunoblotting with biotinylated IgD/streptavidin as described in materials and methods. Lane 1: IgD-R cross-linking; lane 2: PMA + ionomycin; lane 3: CD3 cross-linking; lane 4: IgD-R cross-linking followed by CD3 cross-linking; Lane 5: Mol. Wt. Std.**

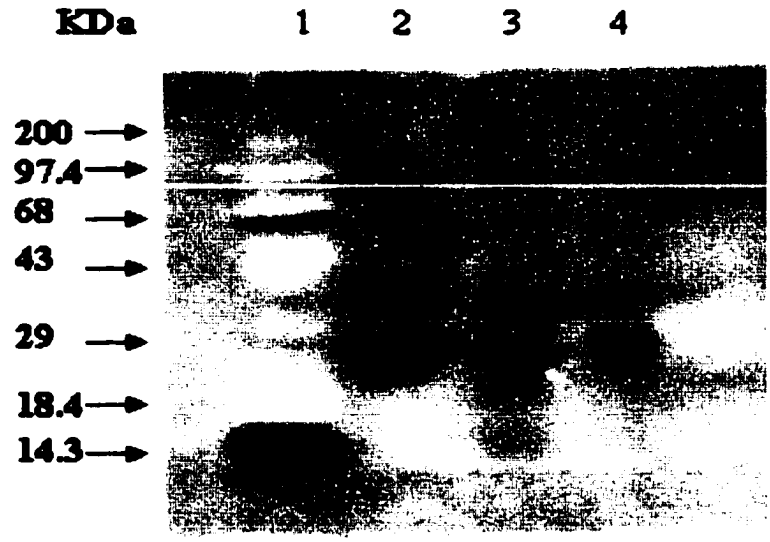

Figure 42. ***IgD-specific binding protein.*** 7C5 cells were stimulated and lysed. Cell lysates were pre-cleared followed by immunoprecipitation with IgD-sepharose beads as described in materials and methods, and analyzed by immunoblotting with biotinylated IgD/streptavivin. Lane 1: Mol. Wt. Std.; lane 2: PMA + ionomycin; lane 3: IgD-R cross-linking; lane 4: medium.

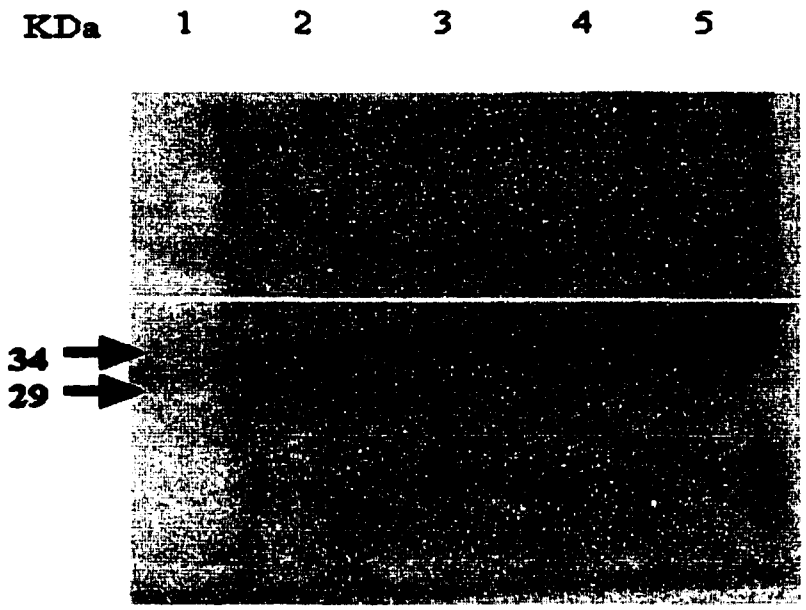

Figure 43. ***Blocking IgD-R and biotinylated IgD interaction by unconjugated IgD.*** **7C5 cells were stimulated and lysed. Cell lysates were pre-cleared, immunoprecipitated with anti-phosphotyrosine antibody coupled to protein G + A-agarose beads. Prior to immunoblotting with biotinylated IgD/streptavidin, blot was incubated in the presence of uncojugated murine IgD. Lane 1: Mol. Wt. Std.; lane 2: medium; lane 3: PMA + ionomycin; lane 4: CD3 cross-linking; and lane 5: IgD-R cross-linking.**

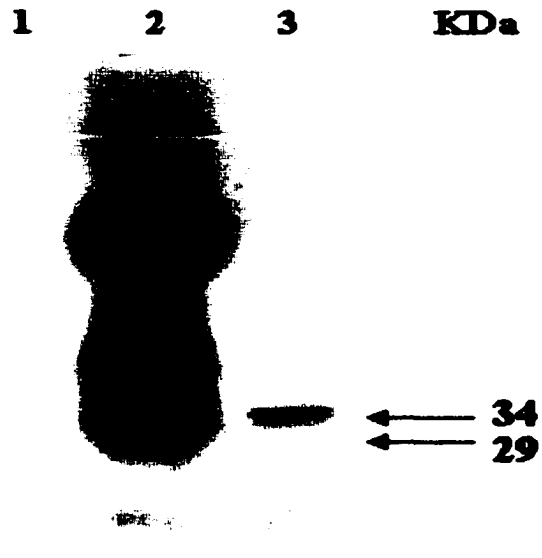

Figure 44. ***Detection of IgD non-specific protein.*** 7C5 cells were lysed and cell lysates were pre-cleared followed by immunoprecipition with IgD-sepharose beads as described in materials and methods, and analyzed by immunoblotting with anti-Ig antibody. Lane 1: Mol. Wt. Std.; Lane 2: mouse Ig; lane 3: IgD immunoprecipitated lysate.

indicate that IgD-R cross-linking induces tyrosine phosphorylation of several intracellular proteins, Moreover, we identified a putative IgD-R or IgD-R component (~29 kDa molecular weight species) on 7C5 T hybridoma cells which appears to be moderately phosphorylated even without stimulation of these cells but which is intensely phosphorylated follwing cell activation.

6. Effects of IgD-R cross-linking on apoptosis in 7C5 cells.

We have shown in our previous studies that IgD-R and IgD interaction play an important role in enhancing antigen-specific antibody production by B cells (Yang and Coico, 1996) and increased proliferative responses by T cells (Wu et. al. 1999). Fas –Fas-L interaction have been shown to send cells death signals and subsequently induce apoptosis (Brunner et. al. 1995; Ju et. al. 1995). In addition, cross-linking of other receptors has been shown to induce upregulation of Fas-Fas-L leading to apoptosis (Brunner et. al. 1995; Ju et. al. 1995). Therefore, we examined whether IgD-R cross-linking induces upregulation of Fas and Fas ligand. 7C5 cells were stimulated by IgD-R cross-linking and, as a positive control, we treated cells with dexamethasone, a known apoptosis-inducing agent. Cells were cultured overnight with these stimuli, washed, and analyzed for Fas and Fas ligand expressions as described in materials and methods. As shown in Figure 45, following overnight culture in dexamethasone-containing medium, there was an increase in double positive staining for Fas/Fas-L when compared with IgD-R cross-linking alone 75% versus 63.7%, respectively, or with medium control (60.6%). Pretreatment with IgD-R cross-linking followed by dexamethasone treatment resulted in lower levels of Fas/Fas-L expression (60.4%) when compared with dexamethasone treatment alone.

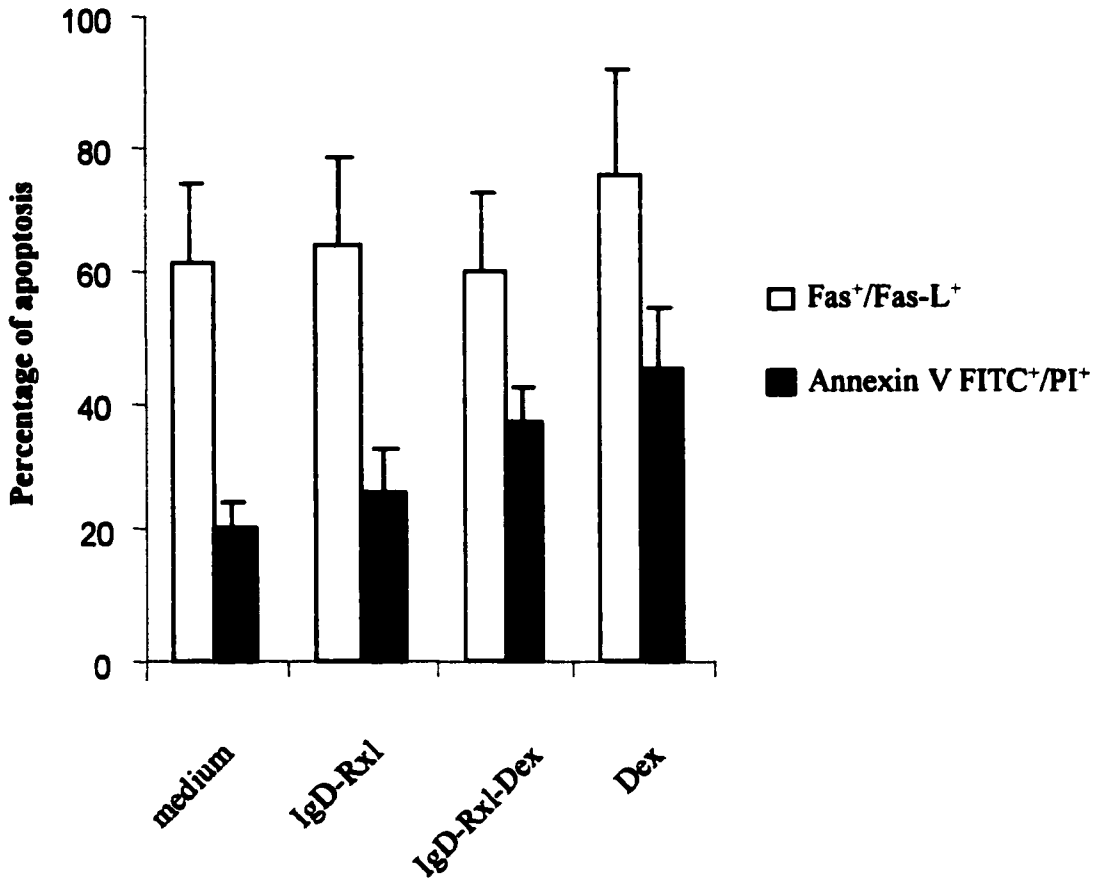

Figure 45. ***Comparison of expression of Fas$^+$/Fas-L$^+$ and late stages of apoptosis.*** **Cells were cultured overnight and analyzed for Fas and Fas-L expression and compared with apoptosis induction (Annexin V-FITC$^+$/PI$^+$) by 72 h culture.**

Fluorescently labelled annexin, a molecule that specifically binds to phosphatidyl serine, allows the detection of cells in early stages of apoptosis (Vermes et. al. 1995). Annexin V-FITC$^+$/PI$^-$ cells were classified as cells in early stages of apoptosis and Annexin V-FITC$^+$/PI$^+$ cells were classified as cells in late stage of apoptosis. As shown in figure 45, in medium alone following a 72 h culture period, about 20% of the cells were in late stages of apoptosis (Annexin V-FITC$^+$/PI$^+$). IgD-R cross-linking caused 25% of the cells to be Annexin V-FITC$^+$/PI$^+$. As expected, dexamethasone treatment resulted in increased apoptosis. By 72 h of culture, the percentage of cells in late stages of apoptosis was significantly higher (44%) than in cells where IgD-R were cross-linked (25%) or in mddium control (20%). Following IgD-R cross-linking, dexamethasone was added to the cells and late stages of apoptosis were analyzed, the percentage of cells in later stages of apoptosis increased to 36% compared with 25% with IgD-R cross-linking alone and to 44% with dexamethasone treatment alone.

Comparrison of Fas/Fas-L expression and late stages of apoptosis in 7C5 cells, the data shows a positive relationship between Fas$^+$/Fas-L$^+$ cells and cells in the late stages of apoptosis, and indicates that ligation of IgD-R with oligomeric IgD (i.e., IgD-R cross-linking) may provide protection against apoptosis induction.

DISCUSSION

IgD is present on the surface of the majority of B cells. It is not secreted following antigenic or mitogenic stimulation of IgD^+ B cells, does not appear to neutralize antigen and does not fix complement. These negative functions support the fact that IgD is primarily a cell-surface antigen receptor (Pernis 1971). *In vivo* studies in mice revealed that IgD injections accelerate the development of germinal centers in lymphoid tissue after birth (Coico et. al. 1988). However, affinity maturation occurring in germinal centers and early IgG1 production in the primary immune response to NP-chicken Ig is delayed in $IgD^{-/-}$ mice. Therefore, in the initiation of the germinal center response, the contribution of IgD to B cell recruitment appears to be particularly important (Roes and Rajewsky 1993; Nitschke et. al. 1993).

The μ and δ heavy chains of IgM and IgD are encoded by a common transcription unit. During B cell development in bone marrow, expression of μ chain on the surface of the late pre-B cells allows their further development to immature B cells. Coexpression of the δ chain and emigration of the immature B cells to the periphery eventually leads to the development of naïve mature IgM/IgD double positive cells. The contribution of IgD in B cell development is still not clear, although IgM is important in driving B cell development (Iglesias et. al. 1993). Studies by Lutz et. al. using mice deficient in IgM ($IgM^{-/-}$ mice) by deleting the μ chain in embryonic stem cells have shown that such mice display normal B cell development and maturation, with IgD replacing membrane-bound and secrectory IgM (Lutz et. al. 1998). Moreover, $IgM^{-/-}$ mice have normal antigen-specific B cell responses and isotype class switching following

immunization or infection. Therefore, early expression of either μ or δ is sufficient to promote B cell development. The μ and δ chains may have similar short cytoplasmatic tail and the ability of Igα and Igβ, the signal-transducting unit of BCR, to associate with all immunoglobulin classes (Reth, 1992). IgD can largely substitute for IgM in driving B cell development, maturation and function suggesting redundancy between these isotypes.

Studies by Xue et al (1984) on the functional property of IgD have shown that elevated oligomeric IgD levels in serum augments the primary and secondary antibody response *in vivo and in vitro*, in normal but not in athymic BALB/c mice. This immunoaugmenting effect can be adoptively transferred by IgD-receptor- positive, $CD4^+$ T cells but not $CD8^+$ T cells or B cells from IgD-treated mice to normal mice (Coico et. al. 1985), suggesting that T helper cell function for antibody production is augmented.

Previous studies from our lab have found that murine $CD4^+$ T cells, but not $CD8^+$ T cells or B cells, can be induced to express a higher density of IgD receptors upon cross-linking of such receptors on their surfaces by oligomeric IgD, antigens, and by exposure to cytokines such as IL-2, IL-4, or INF-γ. In contrast, monomeric IgD neither induces IgD-R^+ Tδ cells nor augments antibody responses. Moreover, studies utilizing $IgD^{-/-}$ mice found that IgD-R can be upregulated by injection of oligomeric IgD, augmentation of antibody production is not observed in such mice. This finding strongly suggests that IgD expression on B cells is required for IgD-R^+ T cells to exert their augmenting effect on antibody responses (Swenson et. al. 1995; Wu et. al.1999). Simultaneous exposure to monomeric and oligomeric IgD prevents IgD-R upregulation

and the augmenting effect of oligomeric IgD on antibody production (Swenson et.al. 1995).

IgD-R has been characterized as having lectin-like properties. Studies utilizing proteolytic fragments of IgD as well as mutant IgD molecules lacking specific C_H domains have shown that in constrast with other Ig-binding FcRs, IgD-R do not bind specifically to the Fc portion of IgD (Tamma et al., 1991; Amin et al., 1991). The interaction between IgD-R and IgD occurs via the recognition of N-glycans on Cδ1 and Cδ3 domains of IgD by IgD-R (Amin et. al. 1991). Structural studies of IgD further demonstrate that the distribution of the carbohydrates on the Cδ-hinge, the density of the carbohydrates on the whole IgD molecules, and the protein sequences of the Cδ regions are important for the interaction between IgD and IgD-R (Yang and Coico, 1996). We suggest that an interaction between surface IgD-associated carbohydrates on B cells and IgD-R on T cells is necessary for immunoaugmentation as evidenced by the fact that addition of monomeric IgD or sugars purified from TEPC1017 or aggregated IgD blocks IgD receptor up-regulation on T cells in vivo and in vitro (Amin et. al. 1991; Swenson et. al. 1995). Immunoaugmentation induced by oligomeric IgD is also abolished by simultaneous injection of monomeric IgD (Swenson et. al.1995).

Our *in vitro* studies have shown that splenic T cells exhibit an increased frequency of IgD-R expression after exposure to IgD by resetting assays and immunofluorescence staining (Coico et. al., 1985c; 1988; Wu et. al. 1999). Since upregulation of IgD-R correlated with increased helper activity of T cells, an increase in affinity of IgD-R for IgD could also explain the effects. We then ask whether affinity of

IgD-R is increased following IgD-R cross-linking. We analyzed IgD-R expression of T cells from IgD treated mice and control mice by using competitive inhibition of IgD-R staining. The results show that cross-linking of IgD-R by oligomeric IgD not only increases the expression of IgD-R on T cells, but also enhances the binding affinity of IgD-R for IgD. We propose that a low number of receptors are present on a significant percentage of normal T cells. Interaction of these IgD-R with sIgD on B cells should be minimal when the IgD on B cells is not interacting with Ag. We hypothesize that cross-linking of sIgD with Ag causes the IgD-R on T cells to become upregulated, allowing for a stronger T-B cell interaction, thus enhancing humoral immune responses.

Our present in vitro studies utilizing ELISPOT assays successfully reproduced and extended previously published studies to assess the functional conquences of B cell interactions with IgD-R$^+$ T cells. We used a hapten-carrier system, and demonstrated that a collaboration between T and B cells resulted from the proximity of carrier-primed T cells and the hapten-primed B cells brought by such an antigen (Mitchison, 1971; Ovary and Benacerraf, 1963). B cells have been found to be an extremely efficient APC when their surface Ig functions to specifically bind antigen with high-affinity binding, therefore focusing the antigen on the antigen-specific T cell (Lichtman et. al. 1987; Tony et. al. 1985). We measured the antibody responses in mice treated with antigen alone, or mice treated with IgD plus antigen. The results showed that injections of oligomeric IgD significantly enhance in vitro primary and early secondary antibody responses to antigen such as TNP-OVA and SRBC. Moreover, studies by using IgD$^{-/-}$ mice showed that oligomeric IgD has no effects on B cell antibody responses, although IgD-R can be upregulated on their CD4$^+$ T cells. The studies strongly suggest that T cells with

upregulated IgD-R and specific TCR significantly enhanced antibody responses to the antigen when B cells express IgD.

In vitro studies of IgD-R$^+$ T cells have shown that the ability of these cells to enhance antibody responses depends upon cognate T-B interactions (Yang and Coico, 1996). Given this requirement for cell-cell contact and the finding that monomeric IgD blocks this effect when added to cultures during the early stages of B cell activation, we have speculated that IgD-R may play a co-stimulatory role in the context of T-B interactions during antigen presentation. Moreover, membrane IgD is rapidly downregulated following antigen stimulation, thereby reducing the chance for effective interaction with IgD-R$^+$ T cells and preventing excessive stimulation of B cells by IgD-R$^+$ T cells. Previously, our efforts have focused on the investigation of functional properties of IgD-R$^+$ T cells with respect to help for antibody responses. In light of the potential for bidirectional signaling following ligation of multiple cell surface receptors during T-B interactions, in the current study, we asked whether antigen-specific T cells also benefit from upregulation of IgD-R when presented with antigen by B cells. We reasoned that if IgD-R expression facilitates the ability of T cells to respond to antigen presented by B cells, then IgD$^+$ B cells presenting Ag should trigger enhanced proliferative responses on the part of IgD-R$^+$ T cells as compared with control T cells which only express low levels of IgD-R. Similarly, if upregulation of IgD-R on IgD-R$^+$ T cells predisposes these cells to receive signals more efficiently from antigen-presenting IgD$^+$ B cells as a consequence of IgD ligation, then proliferative responses by Ag-primed IgD-R$^+$ T cells should be measurably lower when B cells from genetically-deficient IgD$^{-/-}$ mice and APCs other than B cells present antigen. To study cognate T-B cell interaction, amplification of the

initial pool of Ag-specific B cells is important. It has been previously reported that mice bearing a transgenic IgR specific for the antigen can lead to a pool of B cells that express high levels of antigenic complexes and costimulatory ligand, thus fulfilling the requirements as APC for successful T cell priming (Constant et. al. 1995). In our current APC studies, we employed an experimental system, in which goat Ig-specific control T cells and goat Ig-specific IgD-R$^+$ T cells were co-cultured with irradiated B cells as APCs which had been treated in vitro with polyclonal goat anti-IgM or goat anti-IgD. Therefore all B cells served as antigen-presenting cells. The results showed that responses of IgD-R$^+$ T cells were significantly higher than those of control T cells when IgD$^+$ B cells served as antigen-presenting cells. Our current findings support each of these predictions as evidenced by the ability of Ag-primed IgD-R$^+$ T, but not Ag-primed control T cells, to mount augmented proliferative responses to Ag only when IgD is present on the APC population.

It has been demonstrated that IgD-deficient mice are refractory to the in vivo immunoaugmenting effects of oligomeric IgD with respect to antibody responses despite the normal ability of CD4$^+$ T cells from these mice to upregulate IgD-R (Swenson et. al., 1995). In the present study we also demonstrate that cultures of spleen cells from IgD$^{-/-}$ mice containing Ag-primed IgD-R$^+$ T cells fail to show amplified proliferative responses to Ag as compared with control cultures containing Ag-primed IgD-R$^+$ T cells and IgD$^+$ B cells as APCs. Taken together, these findings suggest that interactions between IgD-R$^+$ T and IgD$^+$ B cells during antigen presentation generate bidirectional signals which facilitate both T and B cell activation. In the absence of IgD$^+$ B cells, IgD-R$^+$ T cells with upregulated IgD-R behave like control T cells when stimulated by APCs (B cells or

adherent cells). This conclusion is reinforced by our results showing enhanced stimulation of goat-Ig-primed IgD-R$^+$ T cells by IgD$^{+/-}$ but not IgD$^{-/-}$ antigen-presenting B cells. Furthermore, *in vivo* studies show that when IgD-R$^+$ T cells are induced in IgD$^{-/-}$ mice, antibody responses resemble those of control mice in which IgD-R$^+$ T cells are not induced. Thus, upregulation of IgD-R on Ag-specific T cells only facilitates enhancement of proliferative and antibody responses by T and B cells, respectively, only when these cells interact with B cells expressing membrane IgD. We hypothesize that a cognate mechanism mediated by IgD-R ligation of IgD on B cells is responsible for these bidirectional phenomena.

Several lines of evidence have demonstrated that resting or activated B cells can present antigen to previously activated T cells (Hiramine and Hojo, 1980; Kammer and Unanue, 1980; Chestnut and Grey, 1980; Rock et. al. 1984; Ron and Sprent, 1984) and that activated B cells can activate resting T cells (Krieger et. al. 1985). However, resting B cells are not capabale of presenting antigen to resting T cells (Krieger et. al. 1985; Tony et. al. 1985; Dreiger et. al. 1985). As a major cytokine secreted by macrophages and many other cell types (Durum and Oppenheim, 1989), IL-1 has been found to markely enhance the antigen presentation ability of resting B cells (Kammer and Unanue, 1980) presumably by activating resting CD4$^+$ T cells (Howard and Paul, 1983; Leibson et. al. 1982). Spleen cells employed in most of our experiments were primed with antigen on day-3 relative to the establishment of cultures. Therefore, the B cells involved are newly stimulated from their resting state. IL-1 was therefore a necessary

costimulatory component in our *in vitro* cultures to successfully reproduce the Tδ cell-mediated IgD-induced immunoaugmentation.

An intriguing finding of the present study is the observation that monomeric IgD can inhibit B cell antigen presentation to both Ag-primed control T cells and IgD-R$^+$ T cells implying that low levels of IgD-R expressed on control T cells can also ligate IgD to help regulate these responses. The effect of monomeric IgD on IgD-R$^+$ T cells with upregulated IgD-R cells is explainable since based on studies showing that monomers of IgD inhibit both the ligation of IgD on target cells and the immunoaugmenting properties of these cells (Nitschke et. al. 1993; Swenson et. al. 1995; Yang and Coico 1996). The dose-dependent inhibitory effect of monomeric IgD on Ag-primed control T cells from the current study suggests that basal levels of IgD-R expressed on these cells may also contribute to the ligand/receptor interactions involved in cognate communication with B cells. Thus, as previously shown, monomers of IgD not only appear to cause defective early memory B cell generation in control mice leading to a significant decrease in secondary Ab responses (Swenson et.al. 1995), they also interfere with Ag-specific T cell responses at the initiation of antigen presentation by IgD$^+$ B cells.

The regulatory properties of IgD-R$^+$ T cells conferred by the rapid upregulation of IgD-R on these cells would appear to be limited to the earliest phases of B cell activation. Under physiologic conditions, upregulation of IgD-R by Th cells can occur in response to a variety of stimuli including exposure to certain cytokines (e.g.IL-2, IL-4), IgD-containing immune complexes, and B cells expressing crosslinked membrane IgD (Coico et. al. 1988a). Increased IgD-R expression by CD4$^+$ T cells may transiently

predispose such cells to interact with IgD^+ B cells including precursors of germinal centers and memory cells. Such precursors are known to reside in the $J11D^{low}$ IgD^{high} B cell subpopulation (Linton et. al., 1992; Linton et. al. 1989). Evidence from earlier studies has shown that induction of IgD-R^+ T cells *in vivo* with oligomeric IgD significantly increases germinal center development further supporting the regulatory role of IgD-R^+ T cells (Swenson et. al., 1988). Of interest is the fact that B cells from IgD-deficient mice show delayed affinity maturation, a process which normally occurs within germinal centers (Nitschke et. al., 1993). Thus, the lack of expression of membrane IgD in these mice may lengthen the kinetics of affinity maturation as a consequence of delayed germinal center development.

In summary, our data support the conclusion that IgD-R-IgD ligation contributes to the bidirectional signaling which normally occurs between T and B cells during antigen presentation. T cells engaged in interactions with IgD^+ B cells during antigen presentation appear to benefit from increased expression of IgD-R as evidenced by their enhanced responses to Ag when IgD^+, but not IgD^- B cells, serve as APCs. Similarly, B cells responding to Ag profit from their expression of IgD when cognate interactions with IgD-R^+ T cells occurs by generating significantly increased Ab responses (Xue et. al. 1984; Coico et. al. 1988b; Coico et. al. 1985a; Coico et. al. 1985c). This effect on antibody responses is absent in $IgD^{-/-}$ mice despite the ability of T cells from such mice to upregulate IgD-R expression and display the immunoaugmenting properties associated with IgD-R^+ T cells following adoptive transfer to $IgD^{+/-}$ mice (Swenson et. al. 1995). The proposed ligand/receptor functions of IgD and IgD-R may

help to explain the delayed affinity maturation observed in B cells from $IgD^{-/-}$ mice (Nitschke et. al. 1993).

The synthesis of antigen-nonspecific soluble factors known as cytokines is a key effecter function of the activated $CD4^+$ T cell. The cytokines produced by $CD4^+$ T cells affect the function of multiple cell types, including $CD4^+$ and $CD8^+$ T cells, B cells, macrophages, and the differentiation of bone marrow precursors. Antigen-primed $CD4^+$ T cells can be divided into at least three subsets based the different cytokines they produce. These subsets are known as T_H0, T_H1, T_H2. T_H1 and T_H2 are generated from the antigen-driven differentiation of T_H0 cells, which synthesize IL-2, IFN-γ, IL-4. T_H1 and T_H2 have different effector functions. Cytokines synthesized by T_H1 (IL-2, IFN-γ, TNF-β) are involved in cell-mediated immunity. Cytokines synthesized by T_H2 (IL-4, IL-5, IL-10) trigger B cell proliferation and differentiation. T_H1/T_H2 differentiation has been shown to be influenced by many different factors, such as the cytokine milieu, the nature of the APC involved in response, and costimulatory molecules expressed by interacting cells.

Our studies show that $IgD^{-/-}$ mice lack augmentation of antibody production although IgD-R expression can be upregulated on T cells in this mice. We speculate that IgD and IgD-R interaction facilitates T and B cells activation and enhances antibody responses, perhaps antigen-presenting B cells expressing IgD preferentially activate T_H2 helper cells. Since the functions of helper T cells are predicted by the functions of the cytokines that they synthesize after activation by antigen-presenting cells. IL-4 and IL-5 are the major "helper factors" secreted by T_H2 cells and both act to enhance the growth

and differentiations of activated B cells and induce antibody production (Mosmann and Coffman 1989). Our current results completely support our prediction: cross-linking of IgD-R by oligomeric IgD induces $CD4^+$ T_H2 subtype cytokines IL-4, IL-5 and IL-10 production. In addition, we measured the cytokine mRNA transcription following IgD-R cross-linking further confirmed the regulatory role of IgD and IgD-R on T_H2 cytokine production. Our results indicate that when IgD^+ B cells serve as antigen-presenting cells, IgD and IgD-R interaction may promote T_H2 cell responses. This effect is further confirmed by the finding that cross-linking of IgD-R *in vivo* and *in vitro* by oligomeric IgD simultaneously up-regulates both IgD-R expression and T_H2 subset cytokine production. Our results support the conclusion that interactions between IgD on B cells and IgD-R on T cells enhance T_H2-specific cytokine production and that this ligand/receptor pair may play a costimulatory role to help early differentiation of $CD4^+$ T cell into T_H2 subset.

Costimulatory molecules are critical in enhancing cognate T-B cell interactions. Lack of high enough levels of either MHC class II/peptide complexes or of costimulatory signals, resting T cells cannot be activated by resting B cells. Without activation, the T cell cannot provide the signals needed by the B cell to prolong and successfully complete their interaction (Noelle et. al. 1983). However, it has been hypothesized that physiologic down-regulation of costimulatory molecules on T and B cells following lymphocyte activation prevents severe lymphoproliferative disorders and autoimmune disease (Constant and Bottomly, 1997). A vast amount of evidence suggests that costimulatory signals regulate T and B cells both in terms of normal immune responses and tolerance inductions. Functional studies of IgD have shown that IgD is downregulated after

antigen stimulation. IgD and IgD-R may play a role as costimulatory molecules in T-B cell collaboration.

The interaction of B cell sIgD with IgD-R on T cells could contribute to T and B cell activation, and prolong or strengthen interactions between T and B cells. IgD-R interaction with B cell IgD may also facilitate the induction of the other T-B cell interaction molecules, such as CD28 and CD40L. Our data indicate that CD28 is transiently upregulated on $CD4^+$ T cells following IgD-R cross-linking using oligomeric IgD. This finding provides evidence that interaction between IgD-R on $CD4^+$ T cells with IgD on B cells may play a role in the regulation of lymphocyte responses through CD28-mediated signal transduction.

A variety of ligand/receptor interactions are involved in cognate communication between T and B cells including CD40 and CD40 ligand (CD40L) (Banchereau et. al. 1994) and the B7/CD80/CD86 co-stimulatory molecules with CD28 (June et. al., 1994). Contact-dependent help is also facilitated by interactions between ICAM-1 (CD54) and LAF-1 (Lane et. al. 1991; Poudrier and Owens, 1994), CD30 and CD30 ligand (Shanebeck et. al. 1995), CD27 and CD70 (Kobata et. al. 1995), OX40 and OX40 ligand (Stuber et. al. 1995), and membrane TNFα (Macchia et. al., 1993; Aversa et. al. 1993). Although each of these ligand/receptor interactions may result in unique and often bidirectional signals, there appears to be considerable redundancy in the system to promote T cell-dependent B cell activation. Our data suggest that IgD-R on T_H cells and B cells membrane IgD contribute to this tautological system. A wide range of candidate mechanisms may account for the IgD-R mediated immunoregulatory activity

observed in our system in light of what is known about other co-stimulatory molecules involved in T-B interactions. For example, the CD28-mediated signals result in initiation and maintenance of T cell responses in synergy with TCR-mediated signals; the function of CD28 has been attributed to its ability to enhance the transcription of cytokine genes, to stabilize their messages, to inhibit anergy, and to prevent programmed cell death; and blockade of CD28-mediated signaling can result in T cell tolerance (Bluestone 1995; Green and Thompson 1994; Shi et. al. 1995; Boise et. al. 1995). Furthermore, anti-CD28 induced T cell help is delivered via a CD40L-dependent process (Fassen et. al. 1995). Subsequent CD40-CD40L interaction results in B cell proliferation, differentiation, Ig isotype switching and antibody secretion. The finding that cross-linking of CD40 on B cells promotes expression of the B7 ligand for CD28 confirms that T and B interactions may have a reciprocal amplification mechanism (Klaus et. al., 1994). Other studies suggest that the CD40-CD40L costimulation pathway may allow for selective expansion of $CD4^+$ T cells after interaction with CD40-bearing APC (Cayabyab et. al., 1994). The interactions between LFA-1 and ICAM-1 also play an important role in mediating the collaboration between activated $CD4^+$ T cells and B cells necessary for the induction of B cell proliferation and differentiation, and enhancement of IL-2 production by $CD4^+$ T cells, and that rapid changes in density of ICAM-1 expression and the mobility of ICAM-1 on activated T cells may play a role in providing activation signals to B cells during T cell-B cell collaboration (Tohma S et al 1991,1992). Furthermore, B cells expressing high levels of surface ICAM-1 elicits significantly higher T cell responses than those with low levels suggesting that the expression level of ICAM-1 on peripheral blood B cells correlates with their costimulatory function (Van Kooyk et. al., 1989; Hirokawa et. al.,

1992; Branden and Lundgren, 1993; Hedman and Lundgren, 1992; Denning D etal. 1994). CD30 ligand, a member of the TNF receptor family, was also found to be induced on activated helper T cell clones. Its receptor (CD30) is expressed on freshly isolated and activated murine B cells. Recombinant murine CD30L shares many functional properties with CD40L in the regulation of murine B cell growth and differentiation *in vitro* and may play a role in cognate T-B cell interactions. CD27, another member of the TNF receptor family, binds to its ligand CD70 and induces T cell costimulation and B cell activation. CD27 is expressed on resting T and B cells, whereas CD70 is expressed on activated T and B cells; interactions among subsets of T cells expressing CD27 and CD70 play key role in regulating B cell activation and immunoglobulin synthesis (Kobata T et. al. 1995).

The molecular mechanisms of signal transduction and T cell activation have been studied intensively during the past few years (Perlmutter et. al. 1993; Weiss and Littman 1994; Cantrell 1996; Peterson et. al. 1998). It has become evident that several PTKs play a crucial role. One of the earlier biochemical events seen in T lymphocytes triggered through the TCR complex is the enhanced phosphorylation of number of cellular proteins on tyrosine residues (Perlmutter et. al. 1993; Weiss and Littman 1994; Cantrell 1996; Peterson et. al. 1998). At least two separate signals are required to convert resting T cells into functionally active and responsive T cells. Activation of antigen-specific TCR complex and ligation of co-stimulatory receptors are key steps to T cell clonal expansion and cytokine production (Lenschow et. al. 1996; Bluestone, 1995). Other co-receptor cross-linking mechanisms have been shown to either transmit partial intracellular signals independently or to interfere in TCR/CD3-mediated signals resulting

in anergy or apoptosis induction (Tamma et. al., 1997; Chirmule et. al., 1999). However, unlike CD4 pre-ligation (Newell et. al., 1990; Banda et. al., 1992), pre-ligation of IgD-R with subsequent stimulation through CD3 results in tyrosine phosphorylation of intracellular proteins and suggests that IgD-R do not interfere in TCR/CD3-mediated signals.

Earlier studies have shown that inhibitors of protein tyrosine kinase (PTK) completely prevented upregulation of IgD-R in response to oligomeric IgD suggesting that cross-linking of IgD-R may induce signal transduction and functional consequences through one or more PTK activation pathways (Amin et. al., 1993) leading to upregulation of IgD-R. In the present study we have shown that IgD-R cross-linking by oligomeric IgD indeed results in T cell activation as seen by tyrosine phosphorylation of IgD-R itself and by immunoprecipitation and immunoblotting experiments. Furthermore, a ~ 29 Kda phosphorylated band exhibits strong affinity to biotinylated IgD as has been shown by blocking studies utilizing unconjugated IgD.

IgD-R can be upregulated by triggering T cell surface molecules, such as CD3, CD2, and Thy-1, which signal via one or more second messengers (Altman et. al. 1990; Stefanova et. al. 1991). Previous studies have shown that secondary messenger pathways such as activation and translocation of PKC, increasing levels of intracellular cAMP, changing Ca^{2+} distribution, and activating tyrosine kinase have modulating effects on IgD-R expression. Triggering the CD3 complex of the T cell receptor leads to tyrosine phosphorylation, as well as activation of PKC with an influx of Ca^{2+} (Altman et. al. 1990). T cells triggering via the CD2 pathway induces similar events to those induced by stimulation of TCR-CD3, such as IL-2 secretion, increases intracellular Ca^{2+}, production

of the phosphatidyl inositol pathway-related second messenger and opening plasma membrane Ca^{2+} channel (Altman et. al. 1990; Gardner 1989). Thy-1 signaling on murine T cells involves tyrosine kinase activity and increases intracellular Ca^{2+} (Altman et. al. 1990; Stefanova et. al. 1991). Given our finding that IgD-R can be upregulated as a consequence downstream signaling events associated with CD3, CD2, and Thy1 cross-linking, we speculate that the T cell signaling apparatus is likely to be integrated with this receptor. This integration may represent yet another element of redundancy characteristic of lymphocyte signaling mechanisms. The apparent functional integrity of T cells in $IgD^{-/-}$ mice suggests that IgD-R are not absolutely required in the context of T-B interaction although the delay in class switching seen in such knockout mice may arguably provide evidence to the contrary.

Previous studies of the regulation of IgD-R (Amin et. al., 1993) have examined various signaling inhibitors, including tyrosine kinase inhibitors, herbimycin and genestein; PKC specific inhibitor, Calphostin; non-specific protein kinase inhibitors, staurosporine; cAMP-dependent protein kinase inhibitor, HA1004. They reported that splenic T cells when pre-incubated with genestein or herbimycin A failed to show upregulation of IgD-R by IgD, in contrast to cAMP analogues, and of DiOG + ionomycin or PMA + ionomycin. They also reported that pre-incubation of splenic T cells with relatively non-specific protein kinase inhibitors like stauosporin inhibited the subsequent upregulation of IgD-R by IgD-treatment, however, pre-incubation with calphostin, a more specific PKC inhibitors, did block the upregulation of IgD-R by PMA + ionomycin but not by IgD. The upregulation of IgD-R by anti-CD3 treatment is also inhibited by genestein, herbimycin A, and calphostin. In addition, the upregulation of IgD-R by

increase cAMP is blocked by HA1004. These authors concluded that increasing intracellular cAMP levels and PKC activation each promote upregulation of IgD-R by IgD, but neither of these intracellular signals appears required for IgD-R upregulation by IgD. Based upon the observed regulatory and aspects of IgD-R, there appear to be similarities between this receptor and FcγRII and FcγRIII, both of which show signaling inhibition profiles characteristic of IgD-R (Ravetch and Kinet 1991; Ravetch 1994; Raghavan and Bjorkman 1997). Therefore, like FcγRII and FcγRIII, tyrosine phosphorylation is required for IgD-R upregulation, and IgD-R cross-linking (like FcγR cross-linking) in T hybridoma cells which constitutively express IgD-R, induces tyrosine phosphorylation and T cell activation. These studies further reinforce the conclusion that IgD-R cross-linking may serve as a costimulatory pathway in T cells like cross-linking of CD28 (Norton et. al., 1992; Rieser et. al., 1992), at a lower magnitude.

We further examined the possible role of IgD-R signals that promote apoptosis. Evidence from antigen-presentation studies suggests that the antigen presenting cells (APCs) may dictate the fate of the activated T cells (Brunner et. al., 1995; Ju et. al., 1995). When professional APCs (e.g. dendritic cells) present antigen to naive antigen-specific $CD4^+$ T cells, the responding cells proliferate and secrete cytokines. A significant percentage of these cells also undergo apoptosis. When B cells serve as APCs, a larger percentage of T cells undergo apoptosis (Nagata and Golstein, 1995; Mountz and Zhou et. al., 1995). In the present study, we demonstrated that IgD-R cross-linking on T cells resulted in apoptosis levels that were lower as compared with T cells exposed to an apoptosis-inducing agent (dexamethasone). Specifically, IgD-R cross-linking on T cells,

which are then exposed to dexamethasone resulted in reduced percentage of cells in late stages of apoptosis. These studies suggest that IgD-R cross-linking may protect T cells for receiving death signals only in cells that have been already primed to undergo apoptosis or are in the early stages of apoptosis. In light of our studies using splenic T cells with upregulated IgD-R, which display enhanced proliferative responses when IgD^+ B cells present antigen (Wu et. al. 1999), it is of interest to speculate that upregulation of IgD-R may allow B cells to present antigens without delivering a death signal. We hypothesize that these IgD-R-mediated regulatory mechanisms resulting from ligation of IgD-R (expressed by normal $IgD\text{-}R^+$ T cells) by IgD (expressed by antigen-presenting B cells) may ensure survival of antigen-primed T cells during early activation events *in vivo*. If such a mechanism is at work, the functions of IgD and IgD-R take on new significance. Thus, ligation of IgD-R may predispose antigen-specific T lymphocytes for survival during primary immune responses when B cells serve as APCs.

In summary, the results of this thesis research indicate that: 1) IgD-R and IgD interactions play important regulatory roles in T cell and B cell responses. Upregulated expression of IgD-R on T cells results in enhanced antibody responses to T-dependent antigens. Moreover, we have shown that IgD-R interaction with oligomeric IgD or membrane IgD results in antigen-specific T cell clonal expansion (Wu et. al. 1999). This effect was inhibited when monomeric IgD was added to the cultures. This is in agreement with the notion that IgD-R and IgD may play co-receptor roles similar to other well characterized costimulatory receptor pairs such as CD28-B7 interactions (Lenschow et. al., 1996; Bluestone, 1995). 2) Our cytokine studies shows that cross-linking IgD-R enhances synthesis of cytokines IL-4, IL-5, IL-10 both at the protein and mRNA levels,

suggesting that when B cells act as antigen-presenting cell for T cells expressing IgD-R, this may skew responses toward the T_H2 phenotype. Moreover, cross-linking of IgD-R causes transient upregulation of CD28, a key costimulatory molecule on T cells. 3) Cross-linking of IgD-R on 7C5 T hybridoma cells by oligomeric IgD results in tyrosine phosphorylation of several intracellular proteins. Immunoprecipitation and Western blotting experiments show that the appearance of a ~29 kDa band which is phosphorylated and exhibits strong affinity for biotinylated IgD (putative IgD-R membrane protein). These results indicate that cross-linking of IgD-R leads to activation of one or more protein tyrosine kinase activation pathways. 4) Coincidentally, upregulation of IgD-R and CD69 expression are early events in T cell activation; our present studies have shown that CD69 and IgD-R are two distinctly different molecules and that CD69 appears to exhibit no specificity for IgD. 5) Finally, studies of Fas antigen regulation and expression associated with upregulation of IgD-R suggest that ligation of IgD-R may protect T cells from undergoing apoptosis following their activation by IgD-expressing B cells.

APPENDIX

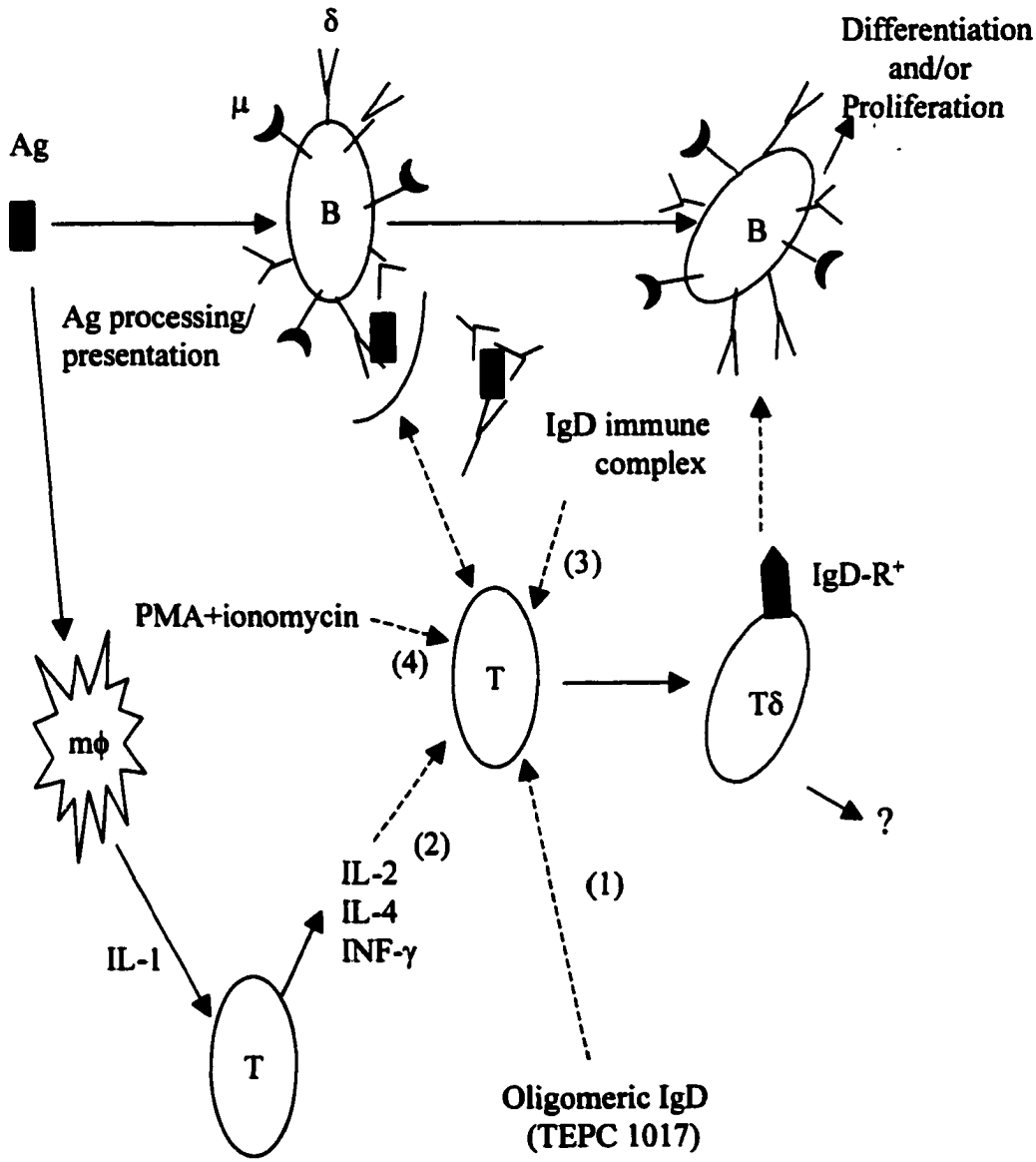

Figure A. Induction of IgD-R+ T cells

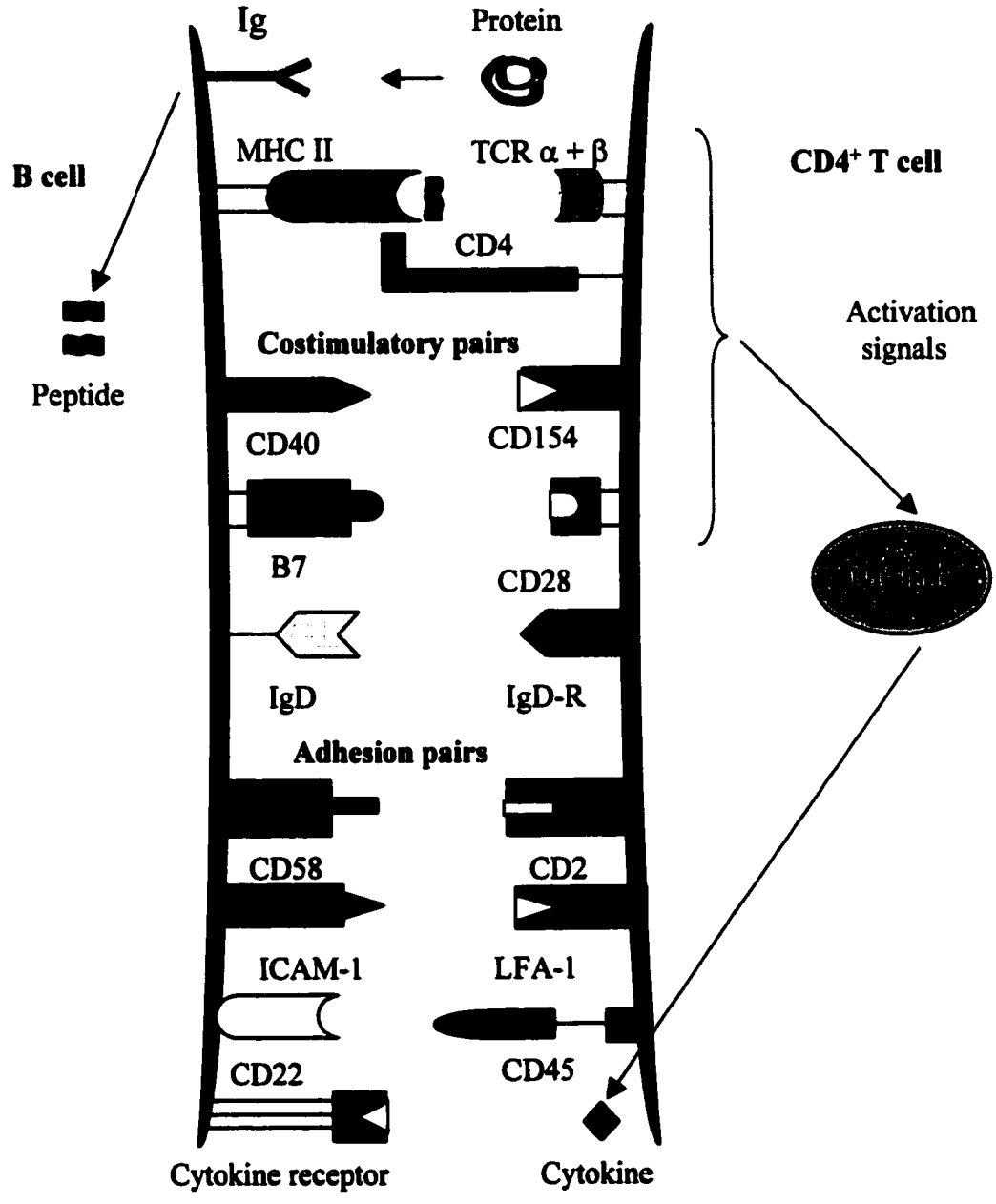

Figure B. Cell surface interactions involved in T-B cooperation

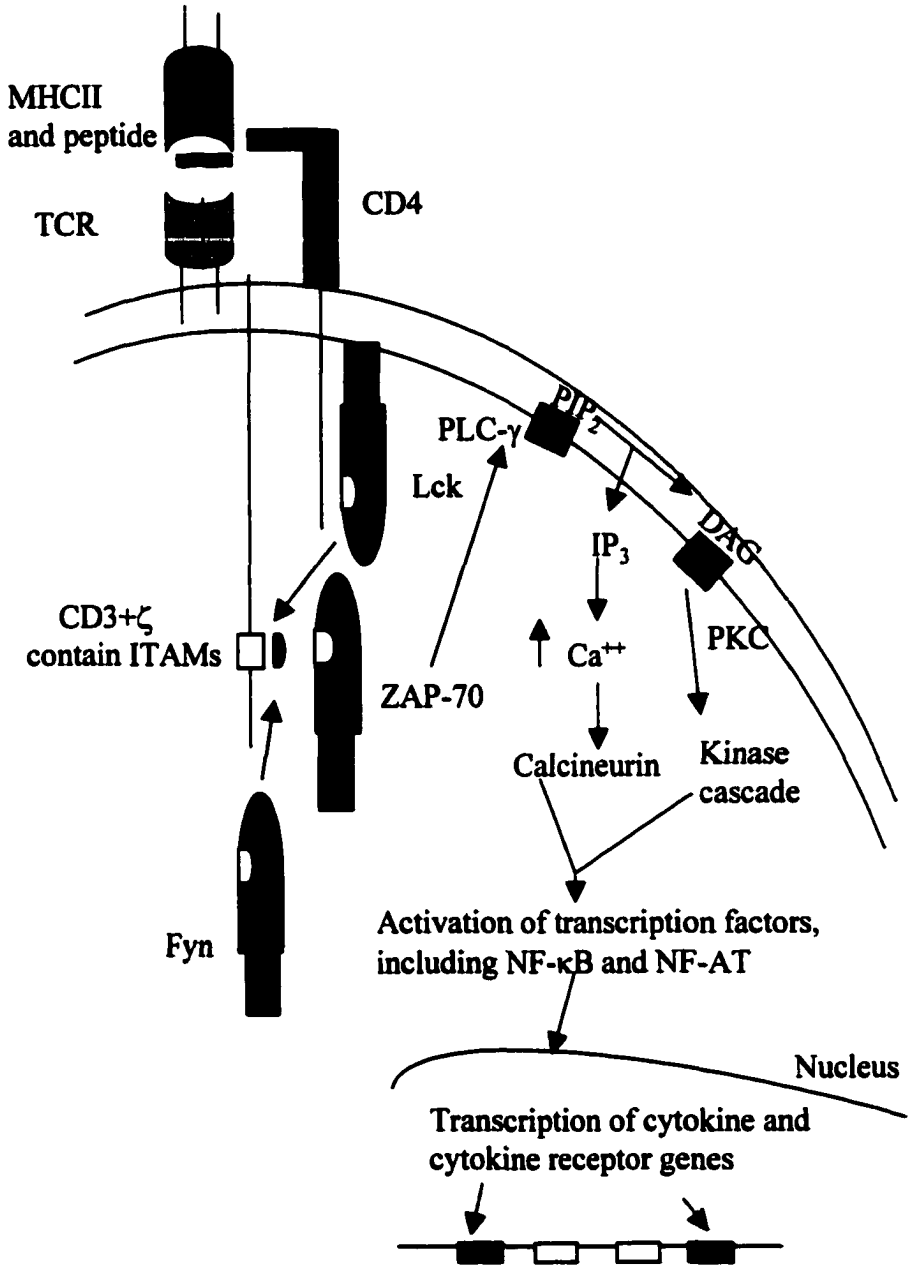

Figure C. Intracellular signaling events in T cell activation

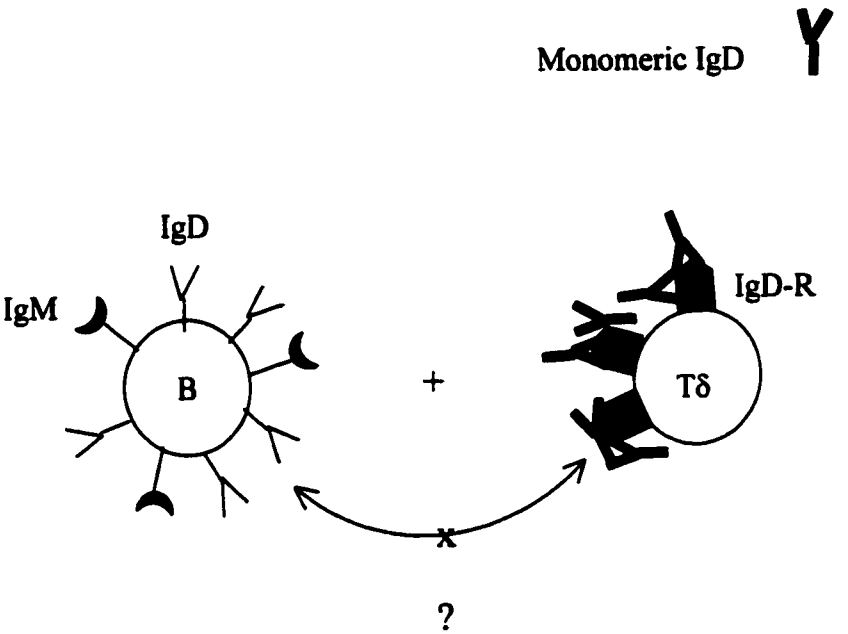

Figure D. Monomeric IgD blocks interaction between IgD-R and membrane IgD on B cells

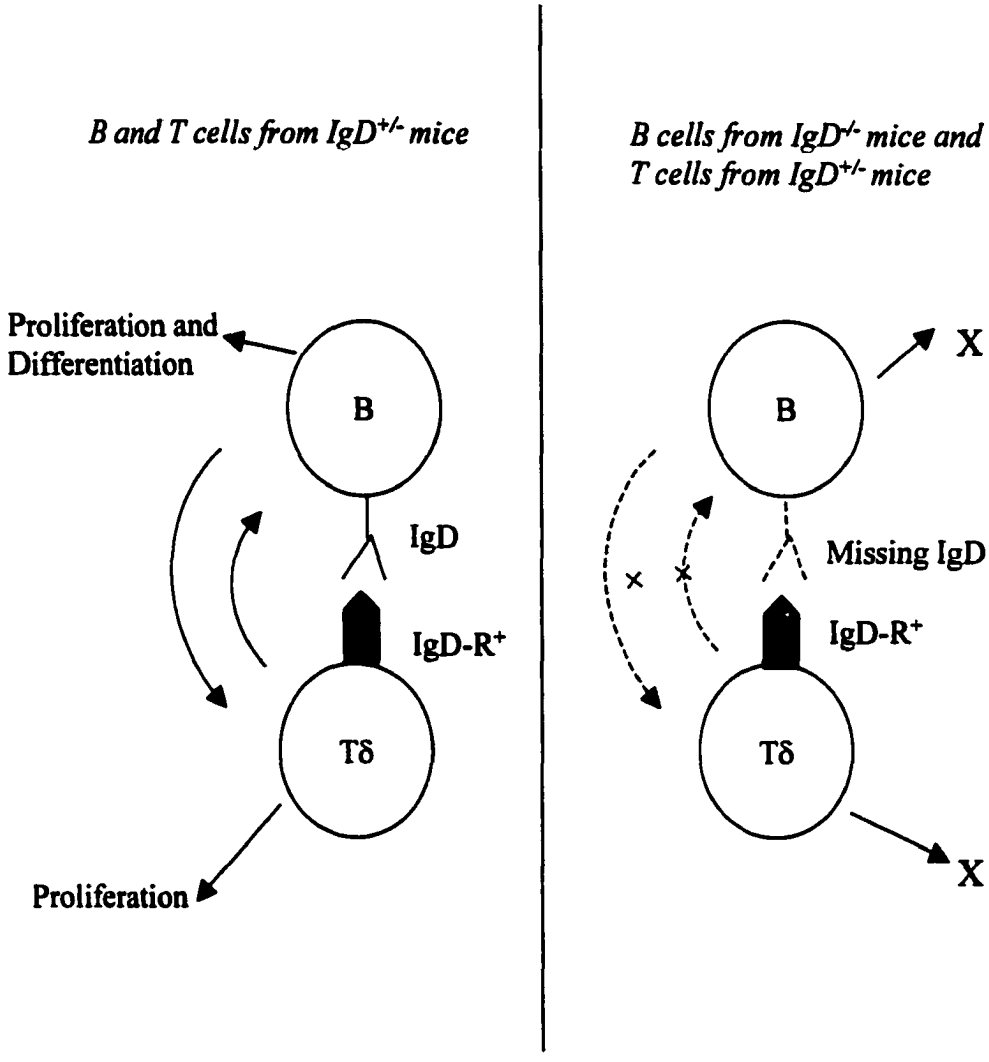

Figure E. IgD/IgD-R mediated bi-directional signaling

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