

FcR EXPRESSION AND FUNCTION IN CD4⁺ T CELL BIOLOGY

By

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FcR expression and function in CD4⁺ T cell biology

To my wife, Cindy

ABSTRACT

Receptors that bind immunoglobulin (Ig) via the Fc domain are known as Fc receptors (FcR). These receptors are integral transmembrane glycoproteins that are expressed on virtually every haematopoietic cell. FcR mediate a wide range of immunological functions from phagocytosis to cellular activation, however, the function and expression of FcR on CD4⁺ T cells is unknown. Thus, *in vivo* and *in vitro* studies were undertaken to investigate FcR expression and function on CD4⁺ T cells.

Previously it has been demonstrated that CD4⁺ T cells activated with antigen-pulsed macrophages or interleukin 1 (IL-1) generated IgG and IgA binding factors (IgGBF and IgABF). These immunoglobulin binding factors (IgBF) are one component of a soluble macromolecule, known as a contrasuppressor factor (CSF). These CSF act to augment IgG or IgA responses *in vivo* and *in vitro* in an isotype-specific manner. Serological and functional data presented here demonstrate that the IgGBF and IgABF are soluble FcγRII/III and FcαR, respectively. sFcR could functionally substitute for the IgBF and enhanced antibody responses in an isotype-specific manner. Recombinant sFcγRII/III, at concentrations of 0.01 to 0.5 ng/ml could augment IgG PFC responses. However, at higher concentrations (10 to 100 ng/ml) it failed to augment IgG PFC responses. Furthermore, the enhancing activity of the IgBF/sFcR was mediated through a subset of T cells that adhered to the lectin *Vicia villosa* (Vv T cells). In *xid* mice, which are unable to generate CSF, T cells could generate the IgBF. However, the *xid* mice lacked circulating regulatory Ig (reg Ig) that is also necessary for the generation of the CSF, resulting in defective CSF.

Since activation of CD4⁺ T cells induced the generation of sFcγRII/III, I investigated FcγR expression on CD4⁺ T cells utilizing an allogeneic activation system. FACS and PCR data from these studies demonstrated that alloactivation of purified CD4⁺ T cells with CH12.LX B cells induced *de novo* FcγRIIB1 expression within 24 hours and prior to DNA synthesis. The induction of FcγRIIB1 expression could be blocked by anti-MHC class II mAb, however, direct TCR ligation, even in the presence of IL-2, was not sufficient to induce expression. Moreover, the induction of FcγRIIB1 expression on CD4⁺ T cells was not dependent upon the Ig isotype of the CH12.LX B cells. Alloactivation of CD4⁺ T cells with different isotype-switch variants of the CH12.LX B cell line resulted in significant increases in the number of FcγRIIB1⁺ CD4⁺ T cells. However, alloactivation with the IgG2b⁺ CH12.LX B cell line induced the largest number of FcγRIIB1⁺ CD4⁺ T cells. Results from this study indicated that the IgG2b⁺ CH12.LX B cell line secreted a soluble factor(s) that augmented FcγRIIB1 expression on alloactivated CD4⁺ T cells. Although, the data suggests that this factor is not soluble IgG2b or IL-1, since both failed to induce FcγRIIB1 expression on purified CD4⁺ T cells.

In conclusion, these data indicate that alloactivation of CD4⁺ T cells induces *de novo* mRNA and surface expression of FcγRIIB1 within 24 hours and prior to DNA synthesis. In addition, CD4⁺ T cell FcγRIIB1 expression can be augmented by a soluble factor(s) secreted by the IgG2b⁺ CH12.LX B cells. Furthermore, activation of CD4⁺ T cells also induces the generation of sFcγRII/III and sFcαR. These sFcR participate in the formation of CSF that enhance antibody responses *in vivo* and *in vitro*, in an isotype-specific manner, through the activation of Vv T cells.

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PREFACE

This dissertation consists of six chapters. Chapter one, the introduction, describes the basic concepts and advances in the field that the thesis deals with and the objectives of the study. Chapters two to five, the core of the thesis, are composed of four individual scientific papers. Each of them addresses a specific facet on the theme of FcR expression and function in CD4⁺ T cell biology. Following these chapters is the summary chapter where the major messages and the overall implications are drawn from the findings presented in the preceding chapters. The references cited in the introduction and summary chapters are listed in the references section at the end of the thesis.

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LIST OF ABBREVIATIONS

ADCC	antibody-dependent cellular cytotoxicity
APC	antigen-presenting cell
ARAM	antigen receptor activation motif
BCR	B cell receptor/surface immunoglobulin
BF	binding factor
CPM	counts per minute
CSF	contrasuppressor factor
EC	extracellular
FcR	Fc receptor
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte/macrophage-colony stimulating factor
GRE	glucocorticoid response element
IC	intracellular
Ig	immunoglobulin
IL	interleukin
Mφ	macrophage
MALT	mucosal associated lymphoid tissue
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
NFκB	nuclear factor κB

NK	natural killer
PBL	peripheral blood lymphocytes
PFC	plaque forming cells
reg	regulatory
sFcR	soluble FcR
SRBC	sheep red blood cell
sup	supernatant
TAM	tyrosine-based activation motif
TCR	T cell receptor
TGF β	transforming growth factor β
T _h	T helper
TM	transmembrane
TNF	tumour necrosis factor
T _s	T suppressor
UTR	untranslated region
Vv	<i>Vicia villosa</i>
Vv T cells	<i>Vicia villosa</i> adherent T cells
<i>xid</i>	X-linked immunodeficiency
YxxL	tyrosine-x-x-leucine

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Chapter One

Introduction

1.1 FcR: an Overview

1.1.1 Cellular distribution

Immunoglobulins (Ig) bind to a group of cellular receptors that interact with the constant region (Fc region) of the Ig heavy chain (Ravetch and Kinet, 1991). These receptors, known as FcR, are isotype-specific and FcR for each of the five Ig isotypes, IgG, IgM, IgE, IgA, and IgD, have been identified in both mouse and man (Ravetch and Kinet, 1991; Nakamura et al., 1993; Daeron and Ishizaka, 1986; Prinz et al., 1987; Daeron et al., 1985; Coico et al., 1985; Sandor et al., 1992c). FcR have been identified on all the major haematopoietic lineages (Ravetch and Kinet, 1991; Daeron et al., 1985; Benhamou et al., 1990; Furuichi et al., 1985; Perussia et al., 1989; Amigorena et al., 1989), excluding all the erythroid lineage cells except for platelets (Karas et al., 1982). Expression of the different classes of FcR (Ravetch and Kinet, 1991; Lynch and Sandor, 1990), the surface density of these receptors (from 10^3 FcR on platelets (Karas et al., 1982) to 10^5 on macrophages (Titus et al., 1984)) and the ontogeny of expression varies greatly between the different cell lineages (Lynch and Sandor, 1990; Rodewald et al., 1992; Foy et al., 1992). Mast cells and other granulocytes (Benhamou et al., 1990; Daeron et al., 1993; Huizinga et al., 1988), natural killer cells (NK cells) (Perussia et al., 1989), macrophages and monocytes (M ϕ) (Weinshank et al., 1988), B cells (Amigorena et al., 1989) and other antigen-presenting cells (APC) (Schmitt et al., 1990) all constitutively express high levels of surface FcR, which can be modulated by cellular activation (Ravetch and Kinet, 1991; Nakamura et al., 1993; Perussia

et al., 1989; Anegón et al., 1988; Weinshank et al., 1988; Daeron et al., 1993; Amigorena et al., 1989). FcR expression on resting T cells is undetectable, however, upon cellular activation, low levels of expression can be detected (Sandor et al., 1992a; Lynch and Sandor, 1990; Sandor and Lynch, 1993; Sandor et al., 1992b; Prinz et al., 1987).

1.1.2 Structure

Fc γ R, Fc ϵ R and Fc α R have been cloned in the human (Ravetch and Kinet, 1991; Kikutani et al., 1986; Brooks et al., 1989), while in the mouse only the Fc γ R and Fc ϵ R have been cloned (Ravetch and Kinet, 1991; Daeron et al., 1990; Ravetch et al., 1986; Hogarth et al., 1991). Molecular and protein analysis of these cloned receptors has revealed that the FcR are members of the Ig supergene family (Ravetch and Kinet, 1991; Qui et al., 1990; Ravetch et al., 1986; Hogarth et al., 1991; Lewis et al., 1986). In humans, the low affinity Fc γ R genes and two of the Fc ϵ R genes, the α and γ subunit, are clustered on chromosome 1q22 (Oakey et al., 1992). This region is syntenic to mouse chromosome 1, where the single genes for these receptors are located (Oakey et al., 1992; Seldin et al., 1993; Kulczycki et al., 1990; Qui et al., 1990). Moreover, it has been suggested that due to the tight linkage of these FcR with another ancestral member of the Ig supergene family, the major myelin protein Po, these receptors may be able to directly interact or form complex interactions with other members of the Ig supergene family (Ravetch, 1994; Oakey et al., 1992). This hypothesis is supported by the observations that activation of murine T cells, via the T cell receptor (TCR), induces

co-migration and co-capping of the FcR and TCR (Sandor et al., 1992a; Sandor et al., 1992b). B cell activation, via surface immunoglobulin (BCR), also induces co-migration and co-capping of the FcR and BCR (Dickler and Kubicek, 1981).

All of the FcR, except for FcεRII (CD23), are glycoproteins comprised of an extracellular (EC) ligand-binding region that consists of 2 or 3 Ig domains of the C2 class (Figure 1) (Ravetch and Kinet, 1991). The EC regions of the FcR are highly conserved, within the FcγR family the EC regions are 70% to 95% homologous (Ravetch and Kinet, 1991; Ravetch et al., 1986; Lewis et al., 1986), and between the FcγR and FcεR there is 40% homology (Ravetch and Kinet, 1991).

The high affinity FcR for IgE (FcεRI) (Blank et al., 1989b) and IgG2a (FcγRI, CD64) (Allen and Seed, 1989) bind monomeric Ig, while the low affinity FcR for IgA (FcαR) (Aicher et al., 1990; Sandor et al., 1990b), IgM (FcμR) (Sandor et al., 1992b; Nakamura et al., 1993; Mathur et al., 1988), FcεRII (IgE, CD23) (Ravetch and Kinet, 1991; Daeron and Ishizaka, 1986), IgD (FcδR) (Amin et al., 1988) and IgG1, IgG2b, IgG2a (FcγRII (CD32) and FcγRIII (CD16)) (Ravetch and Kinet, 1991; Daeron et al., 1985), bind complexed or aggregated Ig. Moreover, some FcR also bind non-Ig ligands. For instance, the low affinity FcεRII also binds CD21, a complement receptor (Aubry et al., 1992; Gagro and Rabatic, 1994) and, FcγRII and FcγRIII bind to a non-Ig ligand present on thymic stromal cells (Sandor et al., 1994; Lynch et al., 1995).

The greatest structural diversity between the FcR occurs within the transmembrane

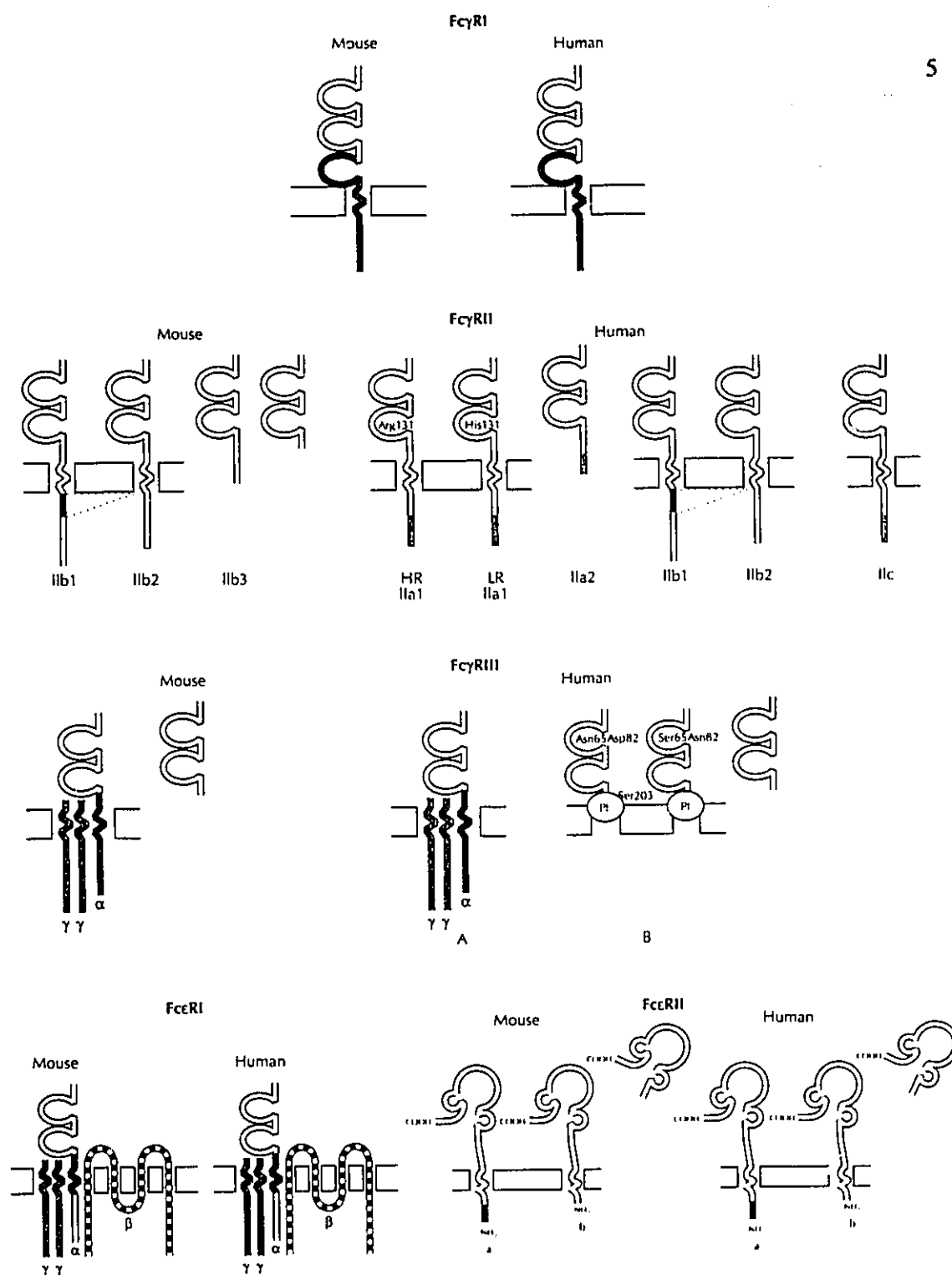


Figure 1. Schematic representations of the murine and human FcR and their associated subunits (From Fridman, W.H., 1993).

(TM) and the intracytoplasmic (IC) domains (Ravetch and Kinet, 1991; Ravetch et al., 1986; Blank et al., 1989b). Moreover, several FcR are also hetero-oligomeric complexes (Figure 1) (Ravetch and Kinet, 1991; Ra et al., 1989; Lanier et al., 1989). These receptors, Fc ϵ RI and Fc γ III, require additional subunits, γ and ζ , for both their assembly and function (Hibbs et al., 1989; Amigorena et al., 1992b; Kurosaki et al., 1991; Wirthmueller et al., 1992; Takai et al., 1994). These subunits are also found associated with the TCR-CD3 complex (Letourneur and Klausner, 1991; Irving and Weiss, 1991; Chan et al., 1992; Orloff et al., 1990). During assembly, γ homodimeric or $\gamma\zeta$ heterodimeric subunits associate with the FcR in the endoplasmic reticulum and protect the receptors from proteolytic degradation (Hibbs et al., 1989; Kurosaki et al., 1991).

The γ or ζ subunits are also essential for mediating the FcR intracellular signals (Eiseman and Bolen, 1992; Wirthmueller et al., 1992). In addition, Fc ϵ RI is associated with a third subunit, β , an integral protein containing four TM spanning regions, which also participates in mediating intracellular signalling (Kuster et al., 1992). Located within the IC domains of Fc ϵ RI, Fc γ RI, Fc γ III and the γ , ζ , and β subunits, are antigen receptor activation motifs (ARAM) (Weiss and Littman, 1994). The ARAM, also known as tyrosine-based activation motifs (TAM), consist of two YxxL sequences separated by 7 to 12 amino acids (Reth, 1989; Keegan and Paul, 1992; Samelson and Klausner, 1992; Malissen and Schmitt-Verhulst, 1993) (Figure 2). This motif is also present within the IC domains of both the α and β Ig chains (Desiderio, 1992) and the δ and ϵ subunits of CD3 (Reth, 1989; Keegan and

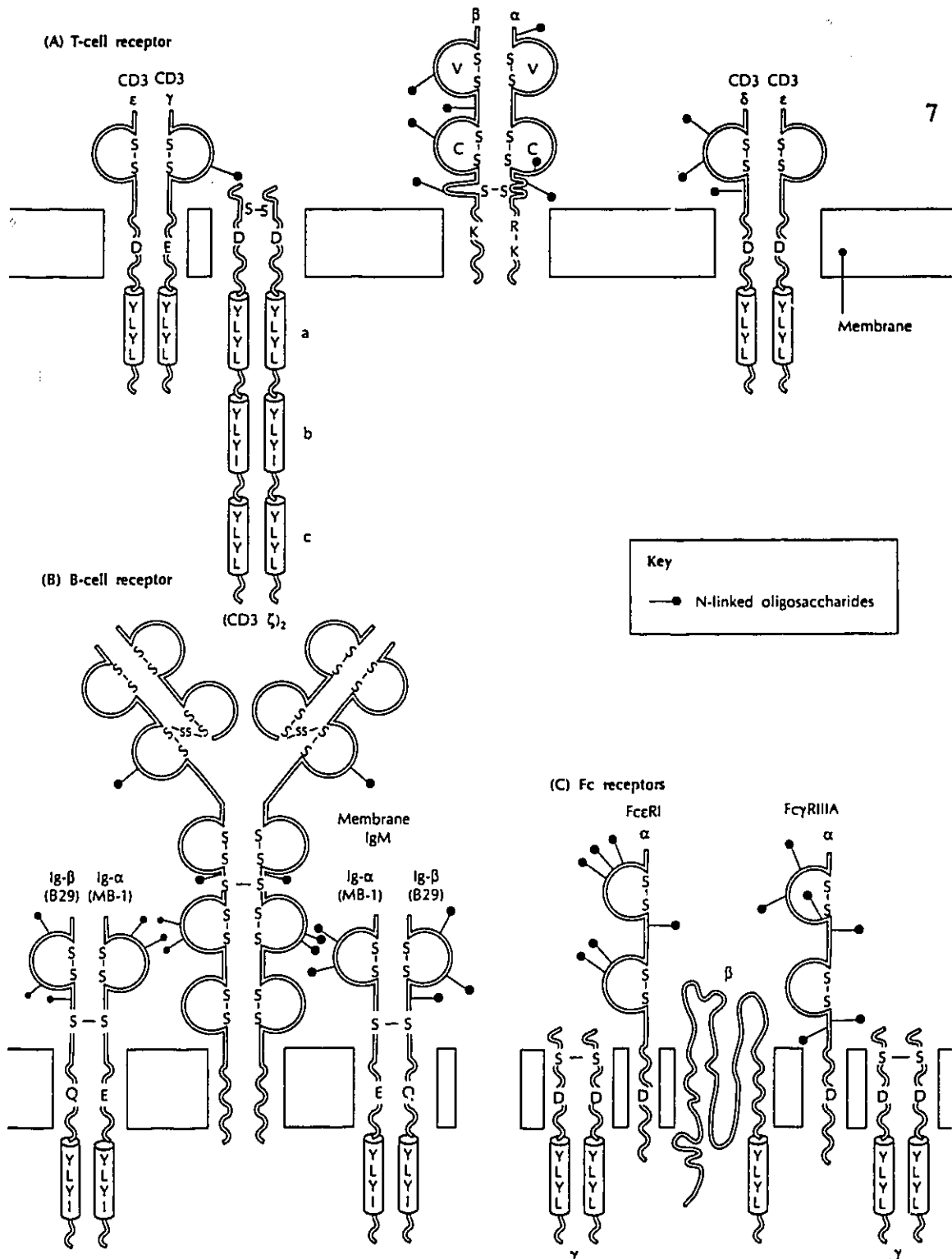


Figure 2. Schematic representations illustrating the ARAM or associated ARAM in members of the Ig supergene family; (A) T cell receptor; (B) B cell receptor/surface immunoglobulin; (C) Fc receptors. ARAM, the YxxL/I motif, are depicted as cylinders in the cytoplasmic domains. (From Malissen, B. and Schmitt-Verhulst, A.-M., 1993).

Paul, 1992; Malissen and Schmitt-Verhulst, 1993) (Figure 2).

1.1.3 FcR signalling

The cross-linking of FcR on most haematopoietic cells results in cellular activation and the induction of a pleiotropy of cellular functions. These cellular responses are similar to the responses observed when the TCR or BCR are cross-linked on T cells and B cells and this is predominately due to the common ARAM signalling motifs located within the IC domains of the three receptors (Desiderio, 1992; Keegan and Paul, 1992; Weiss and Littman, 1994; Cambier, 1992; Weiss, 1993). In a resting cell, the ARAM is associated with the inactive tyrosine kinase *fyn*, of the *src* family (Sugie et al., 1991; Bolen et al., 1992). Upon aggregation of ARAM-containing FcR, the kinase is activated and it phosphorylates the two tyrosines located in the ARAM (Iwashima et al., 1994). The phosphorylated ARAM then interacts with kinases of the *syk* family, ie ZAP-70, through SH2 interactions (Cambier, 1992; Waksman et al., 1993; Weiss, 1993; Waksman et al., 1993; Songyang et al., 1993). This complex then phosphorylates other intracellular substrates, such as phospholipase C- γ 1 and phosphatidylinositol 3-kinase, which ultimately trigger cellular activation (Irving and Weiss, 1991; Liao et al., 1992).

1.1.4 FcR functions

1.1.4.1 Surface FcR

FcR mediate a broad range of biological activities predominantly due to the diversity of the TM and IC regions. For macrophages, activation of FcγR (CD16, CD32 and CD64) triggers a range of integrated cellular functions: oxidative burst (Yamaoto and Johnston Jr. 1984; Anderson et al., 1986), release of intracellular granulocyte contents (Ragsdale and Arend, 1979; Passwell et al., 1980) and gene activation followed by protein synthesis and cytokine secretion (Debets et al., 1990). The FcR also mediate a large spectrum of functions, phagocytosis of IgG coated particles (Walker, 1977), endocytosis of immune-complexes (Ukkonen et al., 1986), antigen presentation (Manca et al., 1991; Lanzavecchia, 1990) and antibody-dependent cellular cytotoxicity (ADCC) (Walker, 1977; Fanger et al., 1989). The cross-linking of the high affinity FcεRI or the low affinity FcγR (CD16 and CD32) on mast cells results in rapid degranulation (Ishizaka and Ishizaka, 1978; Sylvestre and Ravetch, 1994) and the induction of cytokine production (Latour et al., 1992). In NK cells, activation of FcγRIII (CD16) stimulates cytokine production (Anegon et al., 1988), degranulation (Boros et al., 1991) and also mediates ADCC (Perussia et al., 1989; Einspahr et al., 1991).

1.1.4.2 Soluble FcR

Cellular activation of M ϕ (Tartour et al., 1993; Galon et al., 1995), T cells (Teillaud et al., 1990b; Galon et al., 1995) B cells (Sarmay et al., 1991) and NK cells (Huizinga et al., 1988) can induce the generation of soluble FcR (sFcR). Generation of sFcR can occur via proteolytic cleavage of the transmembrane receptor (Sautes et al., 1991). Cleavage usually occurs within the EC domain close to the cell membrane, resulting in the release of a sFcR that corresponds to the Ig-binding domain (Sautes et al., 1991). sFcR can also be generated through an alternative splicing mechanism (Tartour et al., 1993). Fc γ RIIB3 is an isoform of Fc γ RII and during post-transcriptional modifications, the exon encoding the hydrophobic transmembrane region of Fc γ RII is excised and consequently Fc γ RIIB3 is secreted (Tartour et al., 1993). Functionally, the sFcR bind the same ligands as the membrane bound FcR and they also retain their isotype-specificity (Galon et al., 1995; Daeron et al., 1989). sFcR have been detected in both human and murine sera and activation of the immune system stimulates increases in the total serum levels (Daeron et al., 1989; Galon et al., 1995). In addition, serum levels of sFcR are elevated in both mice and humans bearing B cell tumours (Lynch et al., 1995; Daeron et al., 1989).

sFcR can modulate immune responses (Varin et al., 1989; Ra et al., 1993; Ierino et al., 1993) and B cell Ig production, (Teillaud et al., 1990b; Bich-Thuy and Revillard, 1985) *in vivo* (Ra et al., 1993; Ierino et al., 1993) and *in vitro* (Bich-Thuy and Revillard, 1985; Varin et al., 1989; Teillaud et al., 1990b). These molecules have been shown to block both

primary and secondary antigen-specific B cell responses *in vitro* (Bich-Thuy and Revillard, 1985; Varin et al., 1989) and primary responses *in vivo* (Ishizaka, 1988). The activity of the sFcR is mediated directly upon the B cells, inhibiting Ig synthesis by down regulating transcription of both the Ig heavy and light chain genes (Teillaud et al., 1990b; Roman et al., 1988; Brunati et al., 1990). Furthermore, sFcR have been shown to upregulate isotype-specific antibody responses both *in vivo* (Ishizaka, 1988) and *in vitro* (Kiyono et al., 1985).

1.1.5 Mechanisms regulating FcR expression

Currently, the mechanisms responsible for FcR expression remain unclear and the data to date indicates that the mechanisms may differ from one cell type to another and vary upon the experimental conditions. Several cytokines have been shown to modulate FcR expression on both human and murine cells and cell lines. The interferons (α , β and γ) can upregulate Fc γ R and Fc ϵ R surface expression on T cells (Yodoi et al., 1981; Fridman et al., 1980; Yodoi et al., 1983) and Fc γ R expression on M ϕ (Schreiber et al., 1990; van de Winkel et al., 1990) however, these cytokines decrease Fc α R expression on murine T cells (Yodoi et al., 1983). IL-4 can stimulate an increase in the rate of Fc ϵ R synthesis in murine (Conrad et al., 1987) and human B cells (Defrance et al., 1987) and can induce Fc δ R synthesis and surface expression on murine CD4⁺ T cells (Swenson et al., 1993). Furthermore, IL-4 can inhibit the induction of Fc γ R expression on LPS activated murine B cells by decreasing the levels of steady state Fc γ R mRNA (Laszlo and Dickler, 1988; Snapper et al., 1989).

The ligands for the FcR can also modulate cellular expression. Several studies have demonstrated that elevated levels of Ig, *in vivo* (Adachi et al., 1983; Hoover and Lynch, 1983; Mathur and Lynch, 1986) and *in vitro* (Hoover and Muller, 1985; Daeron et al., 1985), can induce or upregulate FcR expression on T cells (Daeron et al., 1985; Yodoi et al., 1983; Coico et al., 1985), B cells (Lee et al., 1987), macrophages (Daeron and Ishizaka, 1986) and basophils (Furuichi et al., 1985). This upregulation of FcR expression can be mediated via stabilization of the surface FcR (Lee et al., 1987) or through the induction of FcR synthesis (Conrad et al., 1987; Defrance et al., 1987).

1.2 FcγRIIB1

1.2.1 Cellular Distribution of FcγRIIB1

Unlike most members of the FcR family that are expressed by a variety of cell types, expression of FcγRIIB1 is limited to B cells (Ravetch and Kinet, 1991; Lewis et al., 1986) and T cells (Ravetch and Kinet, 1991; Ravetch et al., 1986), excluding γ/δ TCR⁺ T cells that only express FcγRIII (Sandor et al., 1992b; Kuziel et al., 1991). FcγRII surface expression has been detected on both CD8⁺ T cells (Mathur and Lynch, 1986) and T cell lines (Brunati et al., 1990; Ravetch et al., 1986) and on some CD4⁺ T cell lines (Sandor et al., 1990a), however, FcγRIIB1 expression has only been detected in CD8⁺ T cells (Ravetch et al., 1986). FcγRIIB1 is constitutively expressed at high levels in B cells (Amigorena et al., 1989), however, in CD8⁺ T cells its expression is highly regulated and it has been suggested that only

activated T cells express this receptor (Sandor and Lynch, 1993; Sandor et al., 1992a). Moreover, it has been suggested that only Th2 type CD4⁺ T cells express FcR (Sandor et al., 1990a). FcγRIIB1 mRNA and surface expression has also been detected on murine NK/T cell precursor thymocytes (Rodewald et al., 1992; Sandor et al., 1994; Lynch et al., 1995).

1.2.2 Structure of FcγRIIB1

1.2.2.1 Gene

The gene that codes for FcγRIIB1, FcγRII, like its human homologue, is located on chromosome 1 (Kulczycki et al., 1990; Qui et al., 1990) and has been cloned and sequenced (Ravetch et al., 1986; Hogarth et al., 1991). The FcγRII gene contains 10 exons (Figure 3) (Hogarth et al., 1991). Exons 1, 2 and 3 encode the 5' untranslated region (UTR), and in addition, exon 3 also encodes the translation initiation codon and 75 base pairs (bp) of the leader sequence. Exon 4 is only composed of 21 bp and codes for the remainder of the leader sequence. Exons 5 and 6, each encode one extracellular Ig-binding domain. These exons are almost identical in size, 258 and 261 bp, respectively. The coding of individual Ig-binding domains within a single exon is typical of members of the Ig supergene family (Ravetch and Kinet, 1991; Hogarth et al., 1991; Qui et al., 1990; Ravetch et al., 1986; Lewis et al., 1986; Malissen and Schmitt-Verhulst, 1993) (Figure 4). Exon 7 encodes the hydrophobic TM region and 3 amino acids of the cytoplasmic tail. Exons 8, 9, and 10, composed of 141, 38, and 545 bp, respectively, encode the remainder of the cytoplasmic tail. Exon 10 also contains

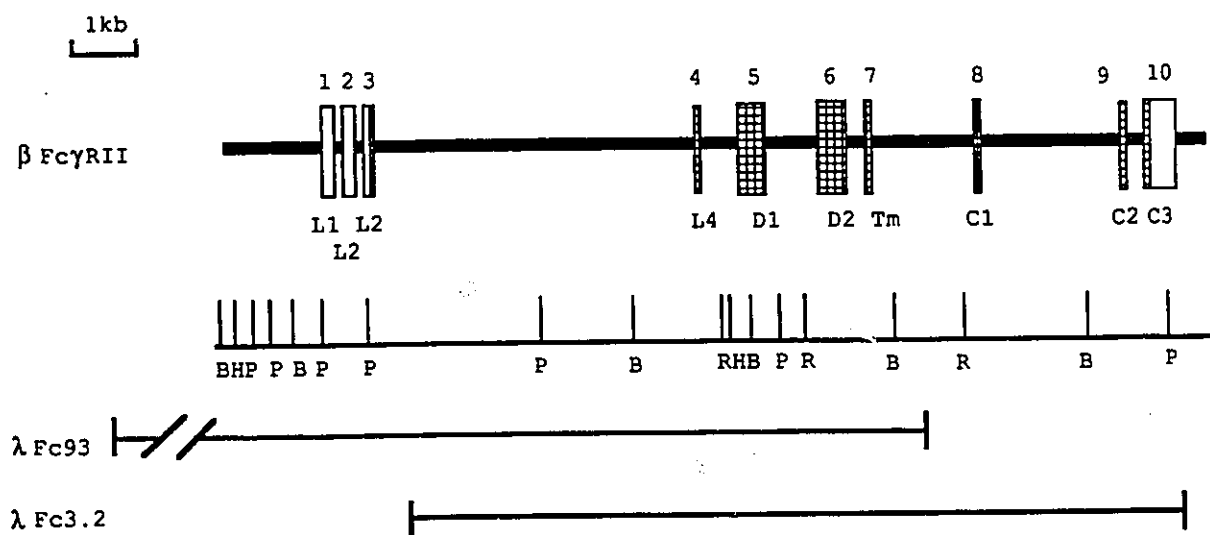


Figure 3. Schematic representation of the exon-intron organization of the murine β Fc γ RII gene. Exons are numbered and indicated by boxes, untranslated regions (UTR) by open boxes and coding regions by filled boxes. Coding regions are also labelled by the region they encode; leader sequences (L), extracellular domains (D), transmembrane regions (Tm) and cytoplasmic tail (C). (From Hogarth, et al., 1991).

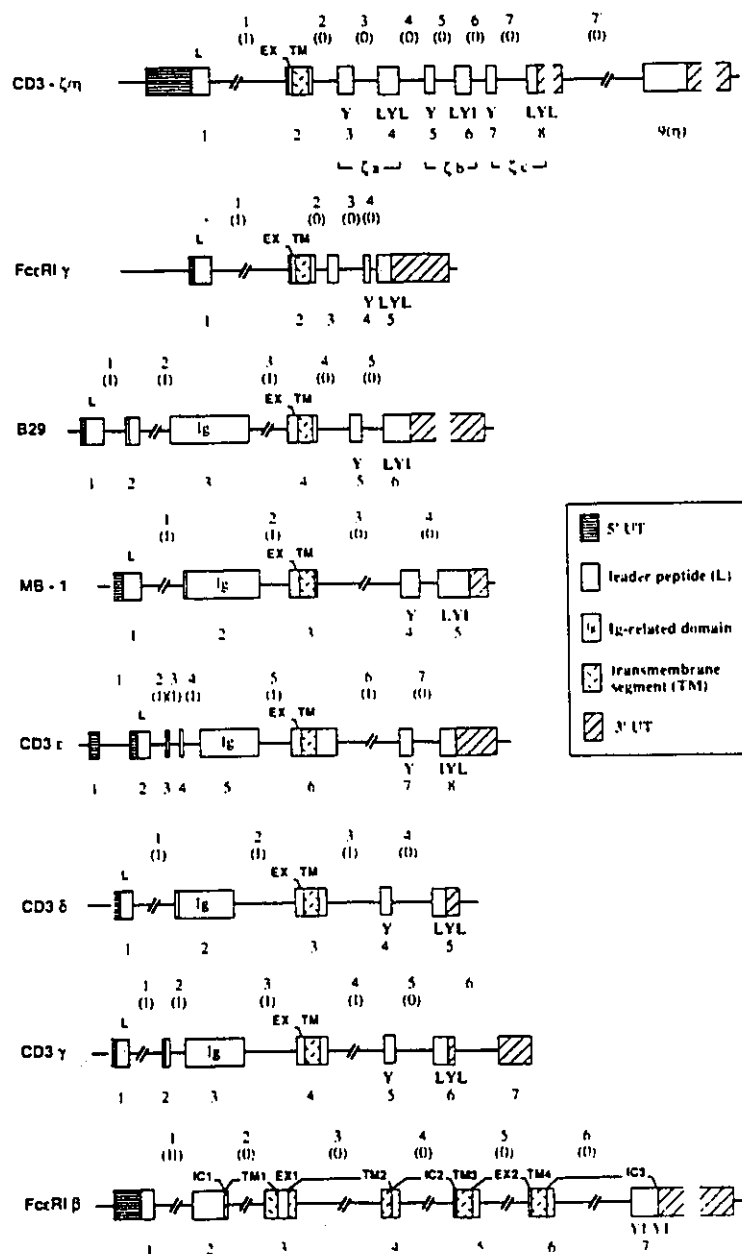


Figure 4. Schematic representation of the exon-intron organization of genes encoding members of the Ig supergene family. Exons are numbered and indicated by boxes and coding regions are also labelled by the region they encode; leader sequences (L), immunoglobulin-related domains (Ig), extracellular regions (Ex), transmembrane regions (TM) and cytoplasmic tail (IC). (From Malissen, B. and Schmitt-Verhulst, A.-M., 1993).

the translation stop codon and most of the 3' UTR (Hogarth et al., 1991).

Several regulatory motifs have also been identified within the gene that may be involved in regulating FcγRII expression (Hogarth et al., 1991; Bonnerot et al., 1988). Two Sp1 sites and one Ap4 site are located 5' of the first exon. In addition, two glucocorticoid responsive elements (GRE) are located upstream and adjacent to the Ap4 site (Hogarth et al., 1991). Three methylation sites have also been identified (Hogarth et al., 1991; Bonnerot et al., 1988). These sites have been shown to be involved in regulating FcγR expression and it has been suggested that these sites may regulate the access of regulatory transcription factors to the DNA (Hogarth et al., 1991; Bonnerot et al., 1988; Doerfler, 1987). In addition, four regions containing alternating pyrimidine/purine nucleotides and a NFκB-like binding site have been located within intron 3 (Hogarth et al., 1991). It has been suggested that these alternating pyrimidine/purine regions may form left-handed helices (Z DNA) and they have been previously mapped near transcriptional enhancer elements (Rich et al., 1984; Ricco et al., 1988).

The FcγRII gene codes for three distinct FcγR, FcγRIIB1, FcγRIIB2 and FcγRIIB3, which are derived from the alternative splicing of the mRNA at the TM and IC exons (Hogarth et al., 1991; Ravetch et al., 1986; Tartour et al., 1993; Ravetch and Kinet, 1991). FcγRIIB1, the largest of the three FcγRII, is generated from mRNA transcript encoding exons 4 to 10 (Ravetch et al., 1986; Hogarth et al., 1991). FcγRIIB2 is generated from a mRNA encoding exons 4 to 7, 9 and 10 (Hogarth et al., 1991; Ravetch et al., 1986) and the

mRNA encoding FcγRIIB3 is comprised of exons 4 to 6, 9 and 10 (Hogarth et al., 1991; Tartour et al., 1993).

1.2.2.2 Protein

FcγRIIB1 is an integral membrane glycoprotein consisting of two extracellular Ig-binding domains, a single membrane spanning domain and an IC domain (Ravetch and Kinet, 1991; Ravetch et al., 1986; Hogarth et al., 1991). FcγRIIB1 is a low affinity FcR ($K_d = 10^6 \text{ M}^{-1}$) that binds complexed or aggregated IgG1, IgG2b and IgG2a (Ravetch and Kinet, 1991). Recently, additional ligands have been identified for this receptor that are present on thymic stromal cells, however, they have yet to be characterized (Sandor et al., 1994). A putative proteolytic site has also been postulated at the base of the EC domain, immediately above the cellular membrane (Sautes et al., 1991).

Within the cytoplasmic tail of FcγRIIB1, amino acid residues 272 to 285, code for the putative coated pit localization domain, which is required for endocytosis (Miettinen et al., 1989; Daeron et al., 1993) and amino acids 285 to 301 are required for phagocytosis (Amigorena et al., 1992a; Daeron et al., 1993; Daeron et al., 1994). However, the 47 amino acid region encoded by exon 8, which is present only in FcγRIIB1 (Ravetch et al., 1986), contains a cytoskeleton attachment site that inhibits FcγRII-mediated endocytosis and phagocytosis (Amigorena et al., 1992a; Daeron et al., 1993; Daeron et al., 1994), thereby preventing B cells from presenting irrelevant/non-specific antigens (Amigorena et al., 1992a).

Furthermore, a 13 amino acid motif located between amino acids 273 to 286, is required for regulating BCR, TCR and FcR signalling (Muta et al., 1994). Interestingly, this regulatory motif is the only sequence that is conserved between the murine and human FcγRII cytoplasmic domains (Brooks et al., 1989).

1.2.3 Mechanisms regulating FcγRIIB1 expression

Currently, little information is known about the mechanisms that regulate FcγRIIB1 expression. As previously stated, expression of the three isoforms of FcγRII is highly restricted (Ravetch and Kinet, 1991; Ravetch et al., 1986; Tartour et al., 1993) and generation of these isoforms occurs at the post-transcriptional level (Ravetch et al., 1986; Tartour et al., 1993; Hogarth et al., 1991). These observations infer that mechanism(s) are present in the various cell lineages which control or direct the post-transcriptional modifications of the newly synthesized FcγRII mRNA, ensuring that only T cells and B cells express the FcγRIIB1 isoform (Ravetch and Kinet, 1991; Ravetch et al., 1986; Lewis et al., 1986).

Cytokines have also been shown to regulate FcγRIIB1 expression. IL-4 has been demonstrated to downregulate FcγRIIB1 expression on both resting (Laszlo and Dickler, 1988) and activated B cells (Snapper et al., 1989) by decreasing the steady state level of FcγRIIB1 mRNA (Snapper et al., 1989). Whole splenic T cell preparations and some CD8⁺ T cell lines, interferon has been shown to upregulate FcγRII surface expression (Yodoi et al.,

1983; Fridman et al., 1980). The ligands for Fc γ RIIB1, IgG1 and IgG2b, have also been shown to induce FcR expression on CD8⁺ T cells *in vivo* (Mathur and Lynch, 1986) and *in vitro* (Daeron et al., 1985). Cross-linking of Fc γ RIIB1 on a CD8⁺ T cell hybridoma has also been shown to upregulate its expression (Daeron et al., 1988). Furthermore, cellular activation of B cells, via LPS (Laszlo and Dickler, 1990; Amigorena et al., 1989) or anti-Ig (Amigorena et al., 1989) and CD8⁺ T cells, via *in vivo* alloactivation (Mathur and Lynch, 1986; Neuport-Sautes et al., 1979) can either upregulate (Laszlo and Dickler, 1990; Amigorena et al., 1989) or induce Fc γ RIIB1 expression (Mathur and Lynch, 1986; Neuport-Sautes et al., 1979; Fridman, 1991; Fridman et al., 1993).

As previously discussed, several regulatory motifs have been located within or 5' to the Fc γ RII gene (Hogarth et al., 1991; Bonnerot et al., 1988). This observation indicates that the cellular expression of Fc γ RIIB1 is highly regulated both positively (Sp1, Ap4, and the NF κ B-like sites) and negatively (GRE site) (Hogarth et al., 1991; Bonnerot et al., 1988; Grilli et al., 1993).

1.2.4 Fc γ RIIB1 signalling

Unlike other FcR, the cross-linking of Fc γ RIIB1 does not induce cellular activation and this is consistent with the observation that it lacks an ARAM motif and is not associated with a subunit that possesses one (Ravetch et al., 1986; Hogarth et al., 1991). However, Fc γ RIIB1 signalling is initiated when cross-linked with a receptor containing, or associated

with, an ARAM (i.e. BCR, TCR, or FcR) (Fridman, 1993; Daeron et al., 1995; Kwack et al., 1995; Daeron et al., 1992; Latour et al., 1992; Daeron et al., 1993; Daeron et al., 1994). This cross-linking results in tyrosine phosphorylation of the YSLL sequence located within the 13 amino acid motif between amino acids 273 to 286 (Muta et al., 1994). Following phosphorylation, Ca^{2+} influx (Wilson et al., 1987; Amigorena et al., 1992a; Bijsterbosch and Klaus, 1985; Choquet et al., 1993) and IP_3 and DAG production (Bijsterbosch and Klaus, 1985) are inhibited and cellular activation and differentiation is arrested (Fridman, 1993; Sidman and Urban, 1976; Klaus et al., 1984; Phillips and Parker, 1984; Phillips and Parker, 1983). It has been suggested that the inhibition of the proliferative signal occurs at an early step in the signal transduction, since tyrosine-phosphorylation of the γ subunit is decreased when Fc ϵ RI are cross-linked with Fc γ RIIB1 (Daeron et al., 1995). Furthermore, competition for tyrosine phosphorylation of other substrate is not observed and Ca^{2+} mobilization of intracellular stores is not perturbed and it is thought that the tyrosine phosphorylation of Fc γ RIIB1 results in the recruitment of a novel SH2 containing protein(s) that ultimately blocks the Ca^{2+} influx (Amigorena et al., 1992a; Muta et al., 1994; Waksman et al., 1993; Songyang et al., 1993). It is also interesting to note that during normal B cell activation, a serine residue located within the IC region of Fc γ RIIB1 also becomes phosphorylated (Hunziker et al., 1990).

1.2.5 Function

1.2.5.1 Surface Fc γ RIIB1

Most of the studies that have been conducted to evaluate the functional role of surface Fc γ RIIB1 have been conducted with B cells. Currently, there is a paucity of data which examines the role of surface Fc γ RIIB1 on T cells. Fc γ RIIB1 appears to negatively regulate B cell function. Cross-linking of Fc γ RIIB1 does not induce cellular activation and seems to have no effect on normal cellular functions (Phillips and Parker, 1983; Dickler and Kubicek, 1981; Bijsterbosch and Klaus, 1985; Daeron et al., 1992; Benhamou et al., 1990). However, when Fc γ RIIB1 is cross-linked with the BCR, a negative regulatory signal is induced that blocks both B cell proliferation and cellular differentiation (Fridman, 1993; Sidman and Uranue, 1976; Klaus et al., 1984; Phillips and Parker, 1984; Phillips and Parker, 1983).

1.2.5.2 Soluble Fc γ RIIB1

In addition to regulating B cell growth and differentiation, Fc γ RIIB1 have also been shown to regulate B cell Ig production (Daeron et al., 1989; Varin et al., 1989; Fridman et al., 1992; Fridman, 1991; Fridman et al., 1993). Soluble Fc γ RIIB1 have been identified in both mouse serum (Lynch et al., 1995) and T cell supernatants (Brunati et al., 1990; Teillaud et al., 1990b). Generation of this soluble form of Fc γ RIIB1 originates through proteolytic cleavage of the transmembrane receptor (Sautes et al., 1991). The cleavage occurs in the EC domain close to the cell membrane and results in the release of a sFc γ RIIB1 that corresponds

to the Ig-binding domain (Sautes et al., 1991). Functionally, the sFcγRIIB1 bind the same ligands as the membrane bound FcγRIIB1 and they retain their isotype-specificity (Sautes et al., 1991; Galon et al., 1995). In mice, the total serum concentration of low affinity soluble FcγR (sFcγR) has been found to be ~150 ng/ml (Lynch et al., 1995; Galon et al., 1995) and activation of the immune system stimulates increases in the total serum levels (Lynch et al., 1995; Daeron et al., 1989; Varin et al., 1989; Galon et al., 1995). In addition, serum levels of sFcγR are 2 to 10 times higher in mice bearing IgG⁺ B cell tumours than in normal mice (Lynch et al., 1995; Daeron et al., 1989; Varin et al., 1989; Galon et al., 1995) and concomitantly, significant increases in FcγRIIB1⁺ CD8⁺ T cells are observed in these mice (Mathur and Lynch, 1986).

FcγRIIB1⁺ CD8⁺ T cells have been shown to regulate B cell Ig synthesis *in vivo* (Mathur and Lynch, 1986) and *in vitro* (Neauport-Sautes et al., 1979; Teillaud et al., 1990a; Blank et al., 1989a; Varin et al., 1989; Fridman et al., 1992) and can block primary and secondary antigen-specific B cell responses *in vitro* (Varin et al., 1989; Fridman et al., 1992; Neauport-Sautes et al., 1979). This activity is mediated through the generation of sFcγRIIB1 that act directly upon the B cells to inhibit Ig production by down regulating transcription of both the Ig heavy and light chains (Teillaud et al., 1990a).

1.3 Purpose of the study

In work previously conducted in Dr. Peter B. Ernst's lab, it was demonstrated that the *in vitro* activation of CD4⁺ T cells, with antigen-pulsed Mφ, stimulated the generation of IgGBF and IgABF that could enhance antigen- and isotype-specific antibody responses *in vitro* (Ernst et al., 1988; Ernst et al., 1989). Furthermore, FcγRIIB1⁺ CD8⁺ T cells have also been shown to generate IgGBF, that have been identified to be sFcγRIIB1, which can regulate B cell Ig production and modulate B cell antibody responses (Teillaud et al., 1990a; Blank et al., 1989a).

Thus, the objectives of this study were:

- 1) to investigate whether CD4⁺ T cells expressed FcR, in particular FcγRIIB1, and
- 2) to determine their role in regulating isotype-specific immune responses.

IgGBF and IgABF participate in the generation of soluble macromolecules that can enhance isotype- and antigen-specific antibody responses in immunologically suppressed systems, *in vivo* and *in vitro* (Ernst et al., 1988; Ernst et al., 1989). Mice with the X-linked immunodeficiency (*xid*) lack this enhancing activity (Braley-Mullen, 1986; Braley-Mullen, 1990). Furthermore, *xid* mice are unable to generate IgG and IgA responses to SRBC (Scher et al., 1979; Slack et al., 1980). Therefore, using both *in vivo* and *in vitro* systems, I investigated whether these defects in the *xid* mice were due a defect in the generation of IgGBF and IgABF by CD4⁺ T cells. The findings from this investigation are presented in the

publication in chapter two.

Previous publications have demonstrated that CD8⁺ T cells can regulate B cell Ig synthesis and modulate antigen-specific antibody responses through the generation of sFcR (Teillaud et al., 1990a; Blank et al., 1989a; Fridman et al., 1992; Galon et al., 1995; Neauport-Sautes et al., 1979). Therefore, I proceeded to investigate whether the IgBF derived from activated CD4⁺ T cells were sFcR. Using anti-FcγRII/III and anti-FcαR specific antibodies and recombinant sFcγRII/III and affinity-purified sFcαR, these studies examined whether CD4⁺ T cell derived IgGBF and IgABF were sFcγRII/III and sFcαR, respectively. The findings from this investigation are presented in the publication in chapter three.

Having observed that activated CD4⁺ T cells generate sFcγRII/III, I employed an allogeneic activation system to define FcγR expression on CD4⁺ T cells. The findings of this study are found in chapter four, which has been submitted for publication and chapter five, which is a manuscript in preparation.

Chapter Two

Role of soluble FcR in the regulation of isotype-specific immune responses

The following article entitled "Mice with the *xid* mutation lack the regulatory antibodies that are necessary for the induction of contrasuppression" is published in Cellular Immunology (164:126-132, 1995). This study investigated the defect that lead to the inability of *xid* mice to generate IgG and IgA anti-SRBC responses.

This work presented in this study was performed by the author of the thesis. This paper was written by the author of this thesis. Dr. Ernst supervised this study.

TITLE:

Mice with the *xid* Mutation Lack the Regulatory Antibodies That Are Necessary for the Induction of Contr suppression.¹

RUNNING TITLE:

Deficiency in regulatory Ig in *xid* mice.

AUTHORS AND AFFILIATIONS:

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77555-0366

Footnotes

1. To whom correspondence should be addressed.
2. This work was supported by grants from the Medical Research Council of Canada.

ABSTRACT

We present evidence that mice with X-linked immunodeficiency (*xid*) lack circulating regulatory immunoglobulin (reg Ig) necessary for control of antigen-specific suppressor T cells (Ts). Previous work demonstrated that reg Ig is one component of a serum factor that blocks Ts activity, thereby allowing appropriate antibody responses *in vivo* and *in vitro*. These factors are referred to as contrasuppressor factors (CSF). CSF are detected in the serum of mice 3-6 hours after immunization with SRBC or can be generated *in vitro* by combining normal mouse serum with supernatants of macrophage-T cell co-cultures (M ϕ -T sup). Data presented here demonstrate that CSF were not detectable in the serum of immunized *xid* mice. Serum from *xid* mice or affinity-purified serum IgG and IgA failed to generate CSF *in vitro*, indicating a lack of reg Ig in *xid* serum. However, *xid* T cells could block suppression of isotype-specific antibody responses *in vitro* when incubated with functional CSF containing M ϕ -T sup and CBA/J reg IgG or IgA. Similarly, *xid* macrophages showed no defect in generation of functional M ϕ -T sup *in vitro*. Finally, CBA/J Vicia villosa adherent (Vv) T cells that were incubated with *in vitro* generated CSF allowed anti-SRBC responses *in vivo*, when adoptively transferred into *xid* mice, prior to SRBC immunization. These responses were comparable to those of normal CBA/J mice immunized with SRBC. Similarly, *xid* mice that received *xid* T cells treated with CSF and were immunized with SRBC generated good anti-SRBC PFC responses. These studies provide strong evidence that *xid* mice lack circulating reg Ig resulting in defective CSF and consequently low antibody responses to SRBC, due to dominant Ts activity.

INTRODUCTION

Previous work has demonstrated that soluble factors can be detected in the serum of mice 3 to 6 hours after immunization with an antigen (1,2). These factors, when passively transferred to tolerized mice, could block antigen-specific suppression (2,3) and could also block antigen-specific T cell mediated suppression *in vitro* (4). The activity of these factors, referred to as contrasuppressor factors (CSF), is mediated directly through a multi-cellular pathway known as the contrasuppressor pathway that blocks T cell mediated suppression of IgG and IgA PFC responses (4,5).

These factors can also be generated *in vitro* by combining normal mouse serum with supernatants from macrophage-T cell co-cultures (6). The CSF generated *in vitro* are identical to the factors found in 6 hour serum because both are soluble macromolecules comprised of IgBF, MHC class II and Ig (7-9). Furthermore, both factors activate the same subset of T cells which adhere to the lectin *Vicia villosa* and the *Vicia villosa* adherent (Vv) T cells block the activity of antigen-specific suppressor T cells *in vivo* and *in vitro* (4).

It has been documented that the contrasuppressor pathway in *xid* mice is non-functional (10,11). We have also observed that the contrasuppressor pathway in *xid* mice was non-functional and these mice failed to generate CSF *in vivo* when immunized with SRBC. Since the contrasuppressor pathway in *xid* mice can be reconstituted through the adoptive transfer of B cells (11), we set out to determine if the defect in the contrasuppressor pathway of the *xid* mice was due to a lack of circulating reg Ig. By varying the murine source of

macrophages, T cells and normal mouse serum used to generate the CSF *in vitro*, we demonstrate that *xid* mice are functionally deficient in circulating reg Ig. Furthermore, we show that anti-SRBC PFC responses by *xid* mice could be reconstituted through the adoptive transfer of Vv T cells incubated with CSF.

MATERIALS AND METHODS

Mice.

Female CBA/J, CBA/Ca, BALB/C and C57Bl/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME). CBA/N (*xid*) mice were also obtained from Jackson Laboratories or from our own colony, established using CBA/N breeding pairs kindly provided by Dr. J. Eldridge from the University of Alabama at Birmingham. All mice used were between 6-8 weeks of age and housed under conventional conditions at McMaster University.

Isolation of murine IgG and IgA.

CBA/J or *xid* serum was adsorbed against anti-IgG or anti-IgA columns. The anti-IgG and anti-IgA columns were generated by conjugating affinity-purified rat anti-IgG or rat anti-IgA (Cedarlane, Hornsby, ON) to cyanogenbromide activated-Sepharose® (Pharmacia, Uppsala), using published protocols (12).

The affinity-columns were first washed with TBS (0.02 M Tris, 0.15 M NaCl, 0.2% Na₃, pH 7.2) and then 2 ml of mouse serum was loaded onto a column and incubated on the column for 1 hr at 4° C. The column was rinsed with TBS and the bound Ig was then eluted with 10 ml of glycine-HCl (0.2 M glycine, 0.15 M NaCl, pH 2.5) and each 1 ml fraction was neutralized with 100 µl of 1 M Tris. The Ig was dialyzed against PBS overnight at 4° C and the Ig was concentrated using Centriprep 30 concentrators (Amicon, Danvers, MA).

Preparation of 6 hour serum.

To generate the CSF *in vivo*, mice were given 5×10^8 SRBC (National Biological Laboratories) i.p. in 250 μ l of PBS and then bled 6 hr later in order to collect the CSF which are present in the serum following immunization (1,2). The blood was allowed to clot for 1 hr at 22° C, spun for 5 min at 1000 g and the serum was collected. To assay for the presence of CSF, 60 μ l of serum was added to suppressed cultures (see below).

Generation of M ϕ -T cell supernatants.

We generated the macrophage-T cell (M ϕ -T sup) supernatants by co-culturing SRBC-pulsed derived macrophages with purified T cells, the supernatants were then subsequently combined with normal mouse serum, as previously described (6,8). For this, freshly isolated *xid* or CBA/J peritoneal cells were incubated in HBSS at 1×10^6 cells per 1 ml of a 1:100 dilution of anti-Thy 1.2 (NEN, Mississauga, ON) for 1 hr at 4° C, and then treated with a 1:10 dilution of rabbit complement (Cedarlane) in HBSS for 1 hr at 37° C. The cells were washed twice with RPMI containing 10% FCS (10% RPMI) and then plated at 8×10^5 cells per ml, in 10% RPMI, in a 2 cm plastic petri dish. 100 μ l of 0.15% SRBC (National Biological Laboratories, Winnipeg, Canada) was added and incubation proceeded for 4 hr at 37° C. Excess SRBC and non-adherent cells were removed by washing with 10% RPMI, at 37° C. Next, 20×10^6 *xid* or CBA/J nylon wool nonadherent splenic T cells, in 1.5 ml of 10% RPMI, were added to the adherent antigen-pulsed macrophage cultures and incubated for 16

hr at 37° C. Functional Mφ-T sup were generated by combining 80 µl of *xid* or CBA/J serum (for a source of regulatory Ig) or affinity-purified CBA/J or *xid* IgG or IgA with 400 µl of Mφ-T sup and incubating for 30 minutes at 37° C (6,8).

***In vitro* Anti-SRBC PFC Generation and Suppression.**

Single cell suspensions of 20×10^6 CBA/J spleen cells were incubated with 100 µl of 0.15% SRBC for 5 days in 1 ml of Eagle's MEM (Gibco), using Marbrook-Diener chambers in order to generate IgM, IgG and IgA anti-SRBC PFC, as previously described (6,8). PFC production in these cultures is inhibited if T_h are added at the beginning of culture (6,8). SRBC-specific T_h were generated *in vivo* by injecting i.v. 100 µl of a 1:10 dilution of anti-I-A^k mAb (H116-32.R5, Cedarlane) into CBA/J mice, followed 3 days later by i.p. injection of 1×10^8 SRBC (in PBS) (6,8). Four days later, spleen cell suspensions from these mice were incubated on nylon wool columns and the nonadherent T cells were eluted and treated with anti-CD5 (7-20.6/3, Cedarlane) plus rabbit complement (Cedarlane). The 5×10^6 of the purified T_h were added to the standard SRBC-PFC culture to suppress IgG and IgA responses. These suppressed cultures could be treated with functional Mφ-T sup to block the T_h cell activity.

Detection of anti-SRBC specific PFC.

Using the Cunningham PFC assay, IgM responses were evaluated as direct plaques and IgG and IgA responses were determined indirectly using monospecific rabbit anti-IgG and anti-IgA (13). IgM responses were subtracted from the indirect PFC values to yield an estimate of IgA and IgG PFC responses. Values for the PFC were expressed as the number of PFC/culture chamber. Data are displayed as mean and SEM for 2-6 experiments.

Isolation and Activation of Vicia villosa adherent T cells.

In some experiments, CBA/J Vicia villosa adherent (Vv) T cells were treated with Mφ-T sup prior to adoptive transfer to *xid* mice or addition to the PFC cultures to inhibit the suppression of PFC responses. The Vv T cells were isolated using a technique previously described (4). Briefly, nylon wool nonadherent splenic T cells in 2% BSA-PBS were plated onto 10 cm plastic petri dishes that had been coated with 250 µg of Vicia villosa (E-Y Laboratories Inc., San Mateo, Calif.), in 5 ml of PBS. Plates were incubated at 4° C for 90 minutes and the nonadherent fraction was gently washed off with PBS at 4° C. Adherent cells were removed by adding 5 mg of N-acetyl-D-galactosamine (Sigma, St. Louis MO) in 10 ml of PBS to each plate and incubating the plates for 20 minutes at 37° C. The dislodged population was then washed 3x before use. The viability of the cells was always >95% as defined by Trypan blue exclusion. For activation, 1×10^5 Vv T cells were incubated with 100 µl of Mφ-T sup for 1 hr at 37° C. The cells were then washed three times and added to

suppressed PFC cultures. 1×10^6 Vv T cells were adoptively-transferred to *xid* mice or 1×10^5 were added to suppressed PFC cultures (see above).

Immunization protocols.

To evaluate *in vivo* anti-SRBC-specific responses, *xid* or CBA/J were given 5×10^8 SRBC (National Biological Laboratories, Winnipeg, Man.) i.p. in 250 μ l of PBS. Seven days later, spleen cells were isolated and tested in a SRBC-specific PFC assay as described above.

RESULTS

***Xid* mice lack serum CSF after immunization with SRBC.**

To determine whether *xid* mice could generate CSF *in vivo*, CBA/J and *xid* mice were immunized with SRBC, bled and the serum was assayed for CSF activity *in vitro*. The 6 hr serum from SRBC immunized *xid* mice showed no CSF activity while 6 hr serum from CBA/J controls had strong CSF activity (Fig 1). Both IgG and IgA anti-SRBC responses were enhanced by CBA/J CSF generated *in vivo*.

Macrophage and T cells from *xid* mice are functional for the generation of the non-Ig components of the CSF, *in vitro*.

To further evaluate the defect in the generation of CSF in *xid* mice, *xid* APC and T cells were examined for their ability to produce the non-Ig components required for CSF *in vitro*. Mφ-T sup were generated using either CBA/J or *xid* macrophage with CBA/J T cells and CBA/J serum. Both *xid* and CBA/J macrophages could be used to generate CSF that enhanced *in vitro* PFC responses, indicating that *xid* macrophages were not defective in this function (Fig 2). A similar CSF activity was obtained when Mφ-T sup were generated using CBA/J macrophage and *xid* T cells, combined with CBA/J serum (Fig 2). Thus, *xid* T cells were not defective in interacting with CBA/J macrophages for generation of the non-Ig components of the SRBC-specific CSF.

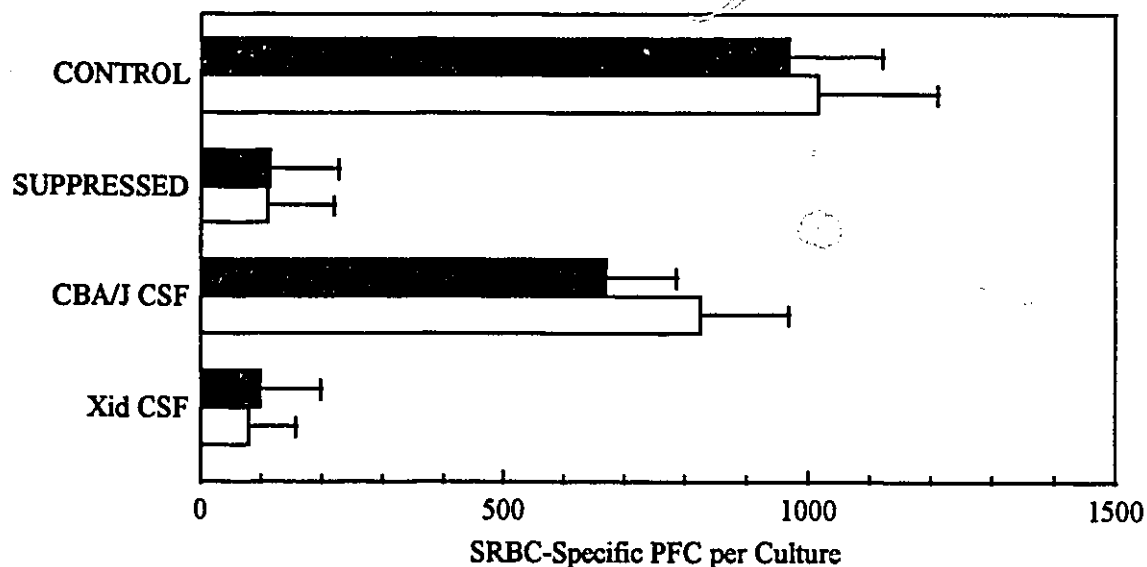


Figure 1 *Xid* mice fail to generate circulating CSF *in vivo*.

In vitro SRBC-specific PFC responses were determined as described in methods. All responder and suppressor T cells were derived from CBA/J mice. CONTROL cultures received only responder cells and SRBC. All cultures except CONTROL received SRBC-specific suppressor T cells (SUPPRESSED). Test cultures were given CBA/J serum (CBA/J CSF) or *xid* serum (XID CSF) collected 6 hr after i.p. immunization with SRBC. Solid data bars represent IgG PFC responses and open data bars represent IgA PFC responses. Data bars indicate mean PFC values from 2 experiments, \pm SEM.

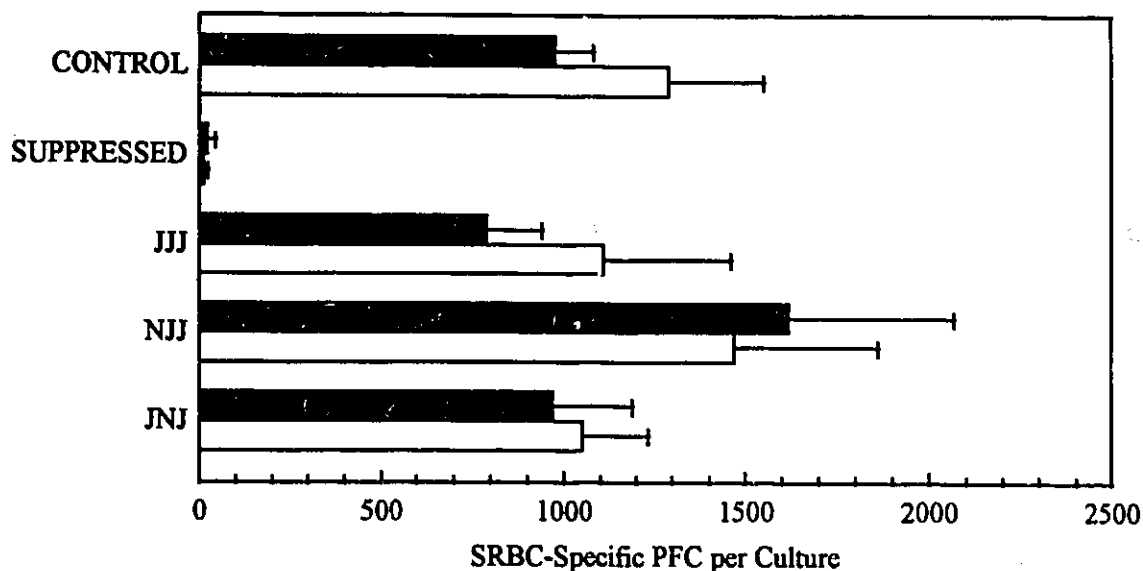


Figure 2 *Xid* APC and T cells were individually examined for their ability to generate the non-Ig components of the CSF.

All cultures including CONTROL and SUPPRESSED were set up as detailed in figure 1 and PFC were determined as described in the methods. Test cultures were given M ϕ -T sup generated with CBA/J APC, T cells and serum (JJJ), *xid* APC, CBA/J T cells and serum (NJJ) or CBA/J APC, *xid* T cells and CBA/J serum (JNJ). Solid data bars represent IgG PFC responses and open data bars represent IgA PFC responses. Data bars indicate mean PFC values from 3 experiments, \pm SEM.

Absence of regulatory Ig in the serum of *xid* mice.

Since the above data indicated that *xid* APC and T cells could participate in the *in vitro* generation of CSF, we determined whether reg Ig was present in the serum of *xid* mice. M ϕ -T sup were generated using either CBA/J or *xid* macrophages and T cells. These supernatants were then incubated with either CBA/J or *xid* serum and assayed for CSF activity. Both CBA/J M ϕ -T sup and *xid* M ϕ -T sup that were combined with CBA/J serum enhanced anti-SRBC PFC responses (Table 1). However, *xid* and CBA/J M ϕ -T sup that were combined with *xid* serum could not enhance PFC responses (Table 1). Neither M ϕ -T sup alone nor CBA/J or *xid* serum alone, could enhance anti-SRBC PFC responses *in vitro* (Table 1).

It has been documented that *xid* mice have abnormally low levels of certain serum Ig isotypes (14). It was possible that the inability of *xid* serum to generate CSF was the result of a quantitative deficiency in Ig rather than a specific reg Ig deficiency. To answer this question, CBA/J and *xid* IgG and IgA were affinity purified. In each case, the IgG was concentrated to 30 mg/ml and the IgA to 5 mg/ml. CBA/J M ϕ -T sup that were combined with CBA/J IgG (final concentration 30 μ g/ml) enhanced only IgG PFC responses, likewise, M ϕ -T sup that were combined with IgA (final concentration 5 μ g/ml) enhanced only IgA responses (Fig. 3). However, CBA/J M ϕ -T sup that were combined with the same concentrations of *xid* IgG or IgA were devoid of any enhancing activity (Fig. 3). The CSF activity was isotype-specific in that M ϕ -T sup combined with CBA/J IgG enhanced only IgG

Table 1. *Xid* serum is deficient in the reg Ig necessary for the *in vitro* generation of CSF.

T_s	M ϕ -T sup ^{a)}		Normal serum ^{b)}		SRBC-Specific PFC no. per culture (\pm SEM) ^{c)}	
	<i>xid</i>	CBA/J	<i>xid</i>	CBA/J	IgG	IgA
-	-	-	-	-	723(94)	675(184)
+	-	-	-	-	75(80)	76(81)
+	-	+	-	-	55(58)	60(64)
+	+	-	-	-	57(60)	42(44)
+	-	-	-	+	68(72)	46(49)
+	-	-	+	-	74(78)	62(66)
+	-	+	-	+	778(100)	1015(221)
+	+	-	-	+	807(145)	894(169)
+	+	-	+	-	64(68)	55(58)
+	-	+	+	-	51(54)	55(58)

a) M ϕ -T sup were generated with *xid* or CBA/J macrophages and T cells.

b) M ϕ -T sup were combined with *xid* or CBA/J serum.

c) Data are mean (SEM) from 3 experiments.

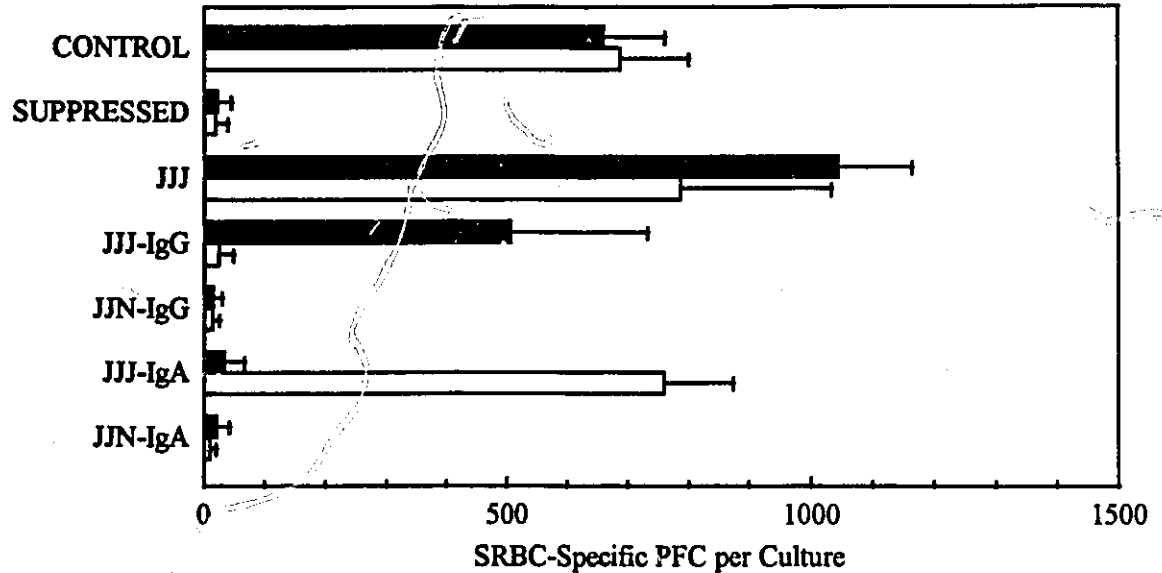


Figure 3 *Xid* IgG and IgA were affinity-purified and concentrated to determine whether reg Ig is present in *xid* serum at low concentrations.

All cultures including CONTROL and SUPPRESSED were set up as detailed in figure 1 and PFC were determined as described in methods. Test cultures received Mφ-T sup generated with CBA/J APC, T cells and either CBA/J serum (JJJ), CBA/J IgG (JJJ-IgG) CBA/J IgA (JJJ-IgA), *xid* IgG (JJN-IgG) or *xid* IgA (JJN-IgA). Solid data bars represent IgG PFC responses and open data bars represent IgA PFC responses. Data bars indicate mean PFC values from 2 experiments, \pm SEM.

PFC responses, likewise, M ϕ -T sup combined with CBA/J IgA enhanced IgA PFC responses.

These results indicated that the deficiency in the *xid* mice was a qualitative feature of the Ig component and that it affected both the serum IgG and IgA fractions. The *xid* reg Ig deficiency was not a quantitative effect because specific reg Ig could not be detected at concentrations of *xid* IgG and IgA that were sufficient to detect reg Ig in CBA/J serum IgG and IgA. Moreover, like CBA/J M ϕ -T sup, *xid* M ϕ -T sup combined with CBA/J IgG or IgA displayed isotype-specific CSF activity. These observations further demonstrated that *xid* T cells and macrophages were not defective and could generate the non-Ig components of CSF for either IgG and IgA responses.

***Xid* T cells have functional contrasuppressive activity after *in vitro* activation with CSF.**

Although our data showed that the *xid* serum was deficient in reg Ig *in vitro*, it was possible that the target cells for these factors (4) were also absent or nonfunctional. We therefore examined whether *xid* Vv T cells could mediate CSF enhancing activity *in vitro*. CBA/J Vv T cells and Vv non-adherent T cells were isolated, incubated with functional CBA/J M ϕ -T sup and then assayed for enhancing activity. Only CBA/J Vv T cells incubated with M ϕ -T sup combined with CBA/J serum enhanced PFC responses (Table 2). As *xid* mice have low numbers of splenic T cells, attempts to purify sufficient numbers of Vicia villosa adherent cells proved difficult. Therefore, we incubated unfractionated *xid* T cells with CBA/J M ϕ -T sup because previous work demonstrated that CSF only activate Vv T cells in

Table 2. *Xid* T cells can interact with M ϕ -T sup to enhance IgG and IgA PFC responses.

Cells Added ^{d)}	Source of Cells ^{e)}	Treatment of Cells ^{a)}	SRBC-Specific PFC ^{b)} no. per culture (\pm SEM) ^{c)}	
		M ϕ -T sup ^{f)}	IgG	IgA
T cells	CBA/J	+	451(192)	847(86)
Vv	CBA/J	-	15(16)	11(12)
Vv	CBA/J	+	455(191)	446(137)
VvNon	CBA/J	-	16(17)	13(14)
VvNon	CBA/J	+	12(13)	10(11)
T cells	<i>xid</i>	-	20(21)	17(18)
T cells	<i>xid</i>	+	407(48)	781(77)

- a) Cells were incubated with M ϕ -T sup for 30 min at 37° C.
- b) Cultures with neither T_h nor M ϕ -T sup had 583(96) IgG and 522(133) IgA SRBC-specific PFC and cultures with only T_h had 10(10) IgG and 15(15) IgA SRBC-specific PFC.
- c) Data are mean (SEM) from 2 experiments.
- d) T cells, *Vicia villosa* adherent (Vv) and non-adherent (VvNon) cells were added to the cultures.
- e) The cells added to the cultures were isolated from CBA/J or *xid* mice.
- f) The M ϕ -T sup were generated with CBA/J T cells, macrophages and serum.

mixed T cell populations (4) (Table 2). Only the *xid* T cells that were incubated with CBA/J M ϕ -T sup combined with CBA/J serum enhanced PFC responses (Table 2). These results indicated that Vv T cells in *xid* mice were functional in the contrasuppressor pathway *in vitro*.

Reconstitution of SRBC responses in *xid* mice by adoptive transfer.

Since our observations indicated that the defect in the contrasuppressor pathway of *xid* mice was due to a lack of reg Ig, we hypothesized that we could reconstitute the anti-SRBC response in *xid* mice through the adoptive transfer of activated Vv T cells. *xid* mice that received CBA/J Vv T cells incubated with CBA/J M ϕ -T sup combined with CBA/J serum generated PFC responses comparable to immunized CBA/J mice, following immunization with SRBC (Table 3). *xid* mice that received *xid* T cells incubated with *xid* M ϕ -T sup combined with CBA/J serum also generated PFC responses comparable to immunized CBA/J mice (Table 3). Furthermore, *xid* mice that received CBA/J Vv T cells incubated with CBA/J M ϕ -T sup combined with CBA/J IgG generated strong IgG responses and when IgA was used they generated strong IgA anti-SRBC responses (Table 3). However, *xid* mice that had received CBA/J Vv T cells incubated with CBA/J M ϕ -T sup, but no Ig, had low PFC responses that were comparable to untreated *xid* mice that had been immunized with SRBC (Table 3).

These results further support the observations that Vv T cells from *xid* mice can mediate CSF enhancing activity. Moreover, like CBA/J Vv T cells, the *xid* T cells could also function in an isotype-specific manner if incubated with CSF containing the necessary reg Ig.

Table 3. Anti-SRBC PFC responses in *xid* mice are reconstituted with the adoptive transfer of Vv T cells activated with CSF containing reg Ig.

Cell Transferred ^{c)}	Source of Cells ^{d)}	Treatment of Cells ^{a)}		SRBC-Specific PFC ^{b)} no. per culture (\pm SEM) ^{e)}	
		M ϕ -T sup ^{e)}	Normal Serum ^{f)}	IgG	IgA
CBA/J ^{g)}	-	-	-	9110(1136)	2837(329)
<i>xid</i> ^{h)}	-	-	-	1537(330)	482(124)
Vv	CBA/J	CBA/J	-	1480(324)	276(81)
Vv	CBA/J	CBA/J	+	8020(891)	2022(241)
T cells	<i>xid</i>	<i>xid</i>	+	6700(959)	1822(264)
Vv	CBA/J	CBA/J	IgG	6480(527)	240(125)
Vv	CBA/J	CBA/J	IgA	536(170)	2534(497)

- a) Cells were incubated with M ϕ -T sup for 30 min at 37° C.
- b) Data are mean (SEM) from 2 experiments.
- c) 1×10^5 T cells, Vicia villosa adherent (Vv) or non-adherent (VvNon) cells were adoptively transferred to *xid* mice 1 hr before immunization with 5×10^8 SRBC.
- d) The cells adoptively transferred to *xid* mice were isolated from CBA/J or *xid* mice.
- e) The M ϕ -T sup were generated with either CBA/J or *xid* T cells, macrophages.
- f) M ϕ -T sup were combined with CBA/J serum or affinity purified IgG or IgA.
- g) CBA/J mice were immunized with 5×10^8 SRBC.
- h) *xid* mice were immunized with 5×10^8 SRBC.

DISCUSSION

Previous work has defined a protein factor in the serum of mice 3 to 6 hours after immunization with SRBC that could block antigen-specific T_H cells *in vivo* and *in vitro* (1-4). These factors, called contrasuppressor factors (CSF), are comprised of Ig binding factors, MHC class II and Ig (7-9). Similar factors can also be generated *in vitro* by combining normal mouse serum with supernatants from macrophage-T cell co-cultures pulsed with SRBC (M ϕ -T sup) (6). The activity of the CSF is indirect because the CSF interact with Vicia villosa adherent T cells (Vv T cells) which inhibit T_H function (4). The CSF allow antigen-specific IgG and IgA PFC responses *in vivo* and *in vitro* in the presence of T_H cells (8,9).

In this present study, we have shown that *xid* mice fail to generate CSF *in vivo*. The serum of *xid* mice, collected 6 hours after immunization, failed to inhibit T_H activity resulting in no IgG or IgA anti-SRBC responses *in vitro*. In contrast, similar serum from CBA/J mice did allow *in vitro* antibody responses.

We next turned to *in vitro* generation of CSF to determine which components may be defective or missing in *xid* CSF. *xid* M ϕ -T sup combined with CBA/J serum supported IgG and IgA PFC responses in the presence of CBA/J T_H cells. However, when the same M ϕ -T sup was combined with *xid* serum, there were no PFC responses. *xid* M ϕ -T sup combined with affinity-purified *xid* IgG and IgA, concentrated to 30 mg/ml and 5 mg/ml, could not enhance PFC responses. However, *xid* M ϕ -T sup combined with similar

preparations of CBA/J IgG or IgA augmented PFC responses. The responses were isotype-specific since CBA/J IgA enhanced IgA PFC, while CBA/J IgG enhanced IgG PFC. This isotype specificity has been reported previously for CSF activity *in vitro* (8,9). These data demonstrated that *xid* mice lacked circulating reg Ig and also showed that *xid* macrophages and T cells could interact together to generate the non-Ig components of the CSF. These observations also precluded the role of cytokines, present in either the CBA/J Mφ-T sup or serum, since neither of these could augment *in vitro* PFC responses by themselves.

Our data indicated that *xid* mice possessed at least one defect in their ability to generate CSF. It was also important to determine whether the cells on which the CSF act were functional. We therefore examined the function of the *xid* Vv T cells, the known target cell of the CSF (4). *xid* T cells treated with CBA/J Mφ-T sup combined with CBA/J serum augmented *in vitro* responses, comparable to responses enhanced by similarly treated CBA/J Vv T cells. Thus, *xid* splenic T cells are capable of inhibiting T_h activity if activated with a proper CSF. Furthermore, the CSF was washed away from the treated T cells, so an indirect effect of CSF through *xid* T cells was observed, as described previously for Vv T cells (4).

Others have documented that *xid* mice fail to generate strong PFC responses to several antigens, including SRBC (15-18). This low responsiveness may result from lack of CSF production and consequently high T_h activity. Our own results indicated that *xid* mice failed to generate CSF *in vivo* when immunized with SRBC. Because our *in vitro* data indicated that the defect in *xid* mice was a lack of circulating reg Ig, we examined whether

xid anti-SRBC PFC responses could be reconstituted *in vivo*, by the adoptive transfer of Vv T cells activated *in vitro* using reg Ig and Mφ-T sup. *xid* mice that received CBA/J Vv T cells incubated with a combination of CBA/J Mφ-T sup and serum, generated strong PFC responses upon immunization with SRBC. This response was comparable to immunized CBA/J mice. *xid* T cells activated with a combination of *xid* Mφ-T sup and CBA/J serum also conferred strong PFC responses following immunization. Furthermore, when CBA/J Vv T cells were incubated with a combination of CBA/J Mφ-T sup and serum IgG and then transferred to *xid* mice, only IgG anti-SRBC PFC responses occurred. Similarly, IgA anti-SRBC PFC responses were reconstituted when *xid* mice received Vv T cells that were first incubated with a combination of CBA/J Mφ-T sup and IgA. These results demonstrate that *xid* mice only require Vv T cells activated with CSF and appropriate reg Ig to produce isotype-specific responses, *in vivo*.

Others have argued that *xid* mice lack contrasuppressor activity due to their deficiency in radiation sensitive B1 B cells because transfer of CBA/J irradiated peritoneal cells to *xid* mice could not restore the SRBC-specific PFC whereas non-irradiated cells could (11). Those authors argued that a lack of antigen-presentation by B1 B cells resulted in the deficiency (11). An equivalent interpretation of those results is that B1 B cells provide reg Ig allowing proper CSF production in reconstituted mice. Our data is consistent with this hypothesis. Our studies also indicated that *xid* adherent peritoneal APC were functional for generation of the non-Ig components of CSF, but do not otherwise comment on antigen

presentation functions by *xid* APC. To restate, we postulate the lack of reg Ig and CSF is directly attributable to the absence of B1 B cells within the *xid* mice (19-21). Within our model, during the initial stages of the immune response, antigen-specific CSF are generated. T cells and macrophage generate a soluble complex of IgBF and MHC class II molecules that combine with reg Ig produced by B1 B cells to form the CSF. The CSF act via the Vv T cells to block the activity of antigen-specific suppressor T cells and do so in an isotype-specific manner.

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Chapter Three

Role of soluble FcR in the regulation of isotype-specific of immune responses

The following article entitled "Soluble FcR block suppressor T cell activity at low concentration *in vitro* allowing isotype specific antibody production" is *in press* in Cellular Immunology. This study investigated whether CD4⁺ T cell derived IgGBF and IgABF were sFcγRII/III and sFcαR, respectively.

Most of the work presented in this study was done, and the paper was written by the author of the thesis. Lenore Zettel conducted three replicate experiments. Dr. Kiyono generously donated the anti-FcαR antisera and the affinity-purified FcαR, Dr. Unkeless donated the recombinant sFcγRII/III and Drs. Snider and Ernst provided supervision to this study, thus resulting in the multiple authorship in this paper.

TITLE:

Soluble FcR block suppressor T cell activity at low concentration *in vitro* allowing isotype specific antibody production. ¹

RUNNING TITLE:

sFcR Regulate the Isotype of Anti-SRBC Responses.

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Footnotes

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ABSTRACT

IgA and IgG binding factors (BF) can be found in the supernatant (T_h sup) of cultures containing macrophages and $CD4^+$ T cells stimulated with particulate antigens such as SRBC. Previous work indicated that these IgBF, when mixed with normal serum immunoglobulin, could block the activity of suppressor T cells (T_s) and allow IgA and IgG PFC responses *in vitro*. We present serologic and functional evidence that IgABF and IgGBF in T_h sup are soluble $Fc\alpha R$ and $Fc\gamma RII$ (or III) respectively. T_h sup adsorbed on affinity columns containing anti- $Fc\gamma RII/III$ mAb or murine IgG, failed to augment IgG PFC responses. Material eluted from either the IgG or anti- $Fc\gamma RII/III$ columns could be added back, interchangeably, to the adsorbed T_h sup and restore IgG PFC. Recombinant murine $Fc\gamma RII$ (r $Fc\gamma RII$), added to the same adsorbed T_h sup at 0.01 to 0.5 ng/ml, resulted in a similar augmentation of IgG PFC. Interestingly, much higher concentrations of r $Fc\gamma RII$ (10-100 ng/ml) could not augment IgG PFC responses. Protein dot blots showed that T_h sup and the eluted material from murine IgG columns contained structures reactive with the $Fc\gamma RII/III$ mAb. Similar studies using purified $Fc\alpha R$ revealed that IgABF eluted from IgA or anti- $Fc\alpha R$ columns was in fact $Fc\alpha R$. Cross-absorption studies indicated clearly that the IgGBF ($Fc\gamma RII/III$) and the IgABF ($Fc\alpha R$) were separate molecules produced in the same T_h sup and that each regulated their respective Ig isotype independently. Thus, cultures of splenic macrophage and $CD4^+$ cells, in the presence of particulate antigens such as SRBC, generate both $Fc\gamma RII/III$ and $Fc\alpha R$. This soluble FcR in combination with serum Ig act to block isotype-specific T_s cells at low concentration *in vitro*.

INTRODUCTION

Different lymphoid tissues have distinct capacities to support the production of various Ig isotypes (1-3). For example, intestinal T cells (especially Peyer's patch T cells) selectively enhance IgA (1,4), while splenocyte T cells preferentially enhance IgM and IgG (1,5,6,7). Two distinct models have been suggested to explain the mechanism of isotype regulation, but they are not yet shown to be mutually exclusive. The more recent model suggests that isotype regulation is a reflection of two distinct cytokine profiles by helper T lymphocytes or other cells within these different tissues that influence B cells directly (8-10). The other model has suggested that isotype regulation is also modulated by isotype-specific suppressor T cells (T_s) and that the expression of FcR by these T_s is important to their function (11-17).

We have been studying the control of isotype-specific T_s *in vitro*, using SRBC as an antigen. Our work (1,5) and that of others (18) indicates that soluble products produced by co-cultures of macrophage and $CD4^+$ T cells *in vitro* can bind to serum IgG or IgA and can block the function of T_s cells in an isotype-specific manner, when combined with serum IgA or IgG. Other data from our laboratory indicates that $CD4^+$ T cells, activated by incubation with recombinant IL-1 alone or antigen-pulsed macrophages, can generate these soluble products (1,5). However, it was not known if the IgBF in the T_s sup were structurally related to FcR.

Murine T lymphocytes express different FcR that bind each of the five Ig isotypes (11-

13) and considerable evidence indicates that these FcR⁺ lymphocytes can regulate antibody isotype both *in vivo* (14,13) and *in vitro* (15-17). Soluble FcR, released by macrophages or lymphocytes have been demonstrated in many recent studies (14-17,19,20). For example, proteolytic cleavage results in release of the B1 form of FcγRII from CD8⁺ T cells and this soluble FcR can mediate isotype regulation by direct inhibition of B cell function *in vitro* (19). In contrast, a CD4⁺ T cell hybridoma, derived from murine Peyer's patch, can preferentially support *in vitro* IgA responses through the production of a soluble FcαR (16,20). Thus, soluble FcR can both positively and negatively regulate antibody responses.

For the present series of studies, we set out to determine clearly if the IgGBF and IgABF generated *in vitro* by CD4⁺ T cells, were related to FcγR and FcαR. Based on antibody specificity for either FcγRII and FcαR, we provide functional and serological evidence that the IgGBF are soluble FcγRII(or III) and that IgABF are soluble FcαR. Further, recombinant FcγRII can act at low concentration to inhibit the T_H cells that control IgG antibody responses, but not IgA antibody responses.

MATERIALS AND METHODS

Mice.

Female CBA/J mice (Jackson Laboratories, Bar Harbour, MA) were used between 8-10 weeks of age and housed under conventional conditions at McMaster University.

Generation of CD4⁺ T cell supernatants (T_h sup)

We generated IgBF in culture supernatants using SRBC-pulsed macrophages co-cultured with CD4⁺ T cells or purified T cells as previously described (23). For this, freshly isolated normal peritoneal cells were incubated in Hank's balance salt solution (HBSS) at 1×10^6 cells per ml containing a 1:100 dilution of anti-Thy 1.2 (NEN, Mississauga, ON) for 1 hour at 4° C. The cells were pelleted, and then treated with a 1:10 dilution of rabbit complement (Cedarlane, Hornsby, ON) in HBSS for 1 hour at 37° C. The remaining cells were washed twice with RPMI containing 10% FCS (10% RPMI) and then plated at 8×10^5 cells per ml, in 10% RPMI, in a 2 cm plastic petri dish. 100 μ l of 0.15% SRBC (National Biological Laboratories, Winnipeg, Canada) was added and incubation proceeded for 4 hours at 37° C. Excess SRBC and non-adherent cells were removed by gentle washing with 10% RPMI, at 37° C. Splenic CD4⁺ T cells were isolated by incubating nylon wool nonadherent spleen cells at 10×10^6 per ml of HBSS containing a 1:300 dilution of anti-CD8 (YTS 169.4, Cedarlane), for 45 min at 4° C. The cells were subsequently incubated with a 1:10 dilution

of rabbit complement (Cedarlane) for 1 hour at 37° C. 20×10^6 of the resulting splenic CD4⁺ T cells, were suspended in 1.5 ml of 10% RPMI and added to the adherent antigen-pulsed macrophage cultures and incubated for 16 hour at 37° C, after which the T_h sup were collected and centrifuged to remove cellular debris.

Adsorption and Elution of IgBF using Ig and anti-FcR Affinity Columns

T_h sup were adsorbed against IgG, IgA, anti-FcγR or anti-FcαR columns. The IgG and IgA columns were generated by conjugating affinity-purified murine IgG or IgA to CNBr-activated Sepharose™ (Pharmacia, Uppsala), using published protocols (24). The mAb 2.4G2 that recognizes FcγRII and FcγRIII (CD32 and CD16) was affinity-purified on a protein-G column from ascites and was used to make the anti-FcγRII column (25). The anti-FcαR column was generated using affinity-purified polyclonal IgG rabbit anti-mouse FcαR produced as described (26).

The affinity-columns (approximately 3 ml packed gel) were first washed with TBS (0.02M Tris, 0.15M NaCl, 0.2% NaN₃, pH 7.2) and then 1.5 ml of T_h sup was loaded onto a column and incubated for 1 hour at 4° C. The initial filtrate was collected in a volume of 5 ml and the column was further rinsed with TBS. The bound material was then eluted with glycine-HCl (0.2 M glycine, 0.15 M NaCl, pH 2.5) and each 1 ml fraction was neutralized with 100μl of 1 M Tris. The eluate was dialysed against PBS overnight at 4° C and then the filtrate (unbound) and the eluate were concentrated to their starting volume using Centriprep

30 concentrators (Amicon, Danvers, MA).

Purified Soluble FcR

A recombinant form of the extra-cellular segment of the Fc γ RII was generated in CHO cells (27) and was a generous gift from Dr. J. C. Unkeless (Mount Sinai, NY). The affinity-purified Fc α R was derived from the T cell hybridoma, Th HA₁ (No. 10) and was isolated from the culture supernatants with an IgA-column (16,20).

***In vitro* Anti-SRBC PFC Generation and Suppression**

Single cell suspensions of 20×10^6 spleen cells were incubated with 100 μ l of 0.15% SRBC for 5 days in 1 ml of Eagle's MEM (Gibco), using Marbrook-Diener chambers in order to generate IgM, IgG and IgA anti-SRBC PFC, as previously described (23). PFC production in these cultures is inhibited if T_h are added at the beginning of culture (23). SRBC-specific T_h were generated *in vivo* by injecting i.v. 100 μ l of a 1:10 dilution of anti-I-A^K mAb (H116-32.R5, Cedarlane) into CBA/J mice, followed 3 days later by i.p. injection of 1×10^8 SRBC (in PBS) (23). Four days later, spleen cell suspensions from these mice were incubated on nylon wool columns and the nonadherent T cells were eluted and treated with anti-CD5 (7-20.6/3, Cedarlane) plus rabbit complement (Cedarlane). Approximately 5×10^6 T_h were added to the standard SRBC-PFC culture to suppress IgG and IgA responses.

These suppressed cultures could be treated with functional T_h sup to block the T_h cell

activity. Functional T_h sup were generated by combining 80 μ l of normal mouse serum (for a source of Ig) with 400 μ l of T_h sup or eluate and incubating it for 30 minutes at 37° C (23). In some experiments, the T_h sup were first adsorbed against Ig or anti-FcR columns and then mixed with IgGBF, IgABF, rFc γ R or affinity-purified Fc α R prior to incubation with serum and use in the PFC culture.

Detection of *in vitro* generated PFC

Using the Cunningham PFC assay, IgM responses were evaluated as direct plaques and IgG and IgA responses were determined indirectly using monospecific rabbit anti-IgG and anti-IgA (28). IgM responses were subtracted from the indirect PFC values to yield an estimate of IgA and IgG PFC responses. Values for the PFC were expressed as the number of PFC/culture chamber. Data are displayed as mean and SEM for 3-6 experiments, except for table 2, where the data is the mean and SD for 3 replicative cultures from one experiment.

Detection of Fc γ RII/III

T_h sup or proteins eluted from anti-Fc γ R and IgG columns were concentrated by a factor of 80 using Centricon 30 concentrators (Amicon) and were blotted onto nitrocellulose paper. The nitrocellulose paper was blocked with 10 mg/ml BSA-TBS blocking solution for 2 hours at 23° C and then washed 3 times in 0.5% Tween-TBS. The blot was then incubated with 1 μ g/ml of anti-Fc γ RII (mAb, 2.4G2), followed by (1 μ g/ml) biotin-conjugated goat

anti-rat IgG (Jackson ImmunoResearch, West Grove, PA), and finally a 1:5000 dilution of extra-avidin conjugated alkaline phosphatase (Sigma, St. Louis, MO). Each of these incubations used 1 mg/ml of BSA-TBS as diluent and were for 2 hours at 23° C, with a 3 times wash step using Tween-TBS, in between each step. The blot was developed with a solution of 0.30 mg/ml NBT and 0.15 mg/ml BCIP in 30 ml of carbonate buffer (0.1 M NaHCO_3 , 1.0 mM MgCl_2 , pH 9.8).

RESULTS

Antibodies to Fc γ RII and Fc α R recognize soluble factors that promote IgG and IgA PFC *in vitro*

In order to evaluate whether the T_h sup contained factors that were structurally related to Fc γ R and promoted IgG PFC, we adsorbed the T_h sup against an anti-Fc γ RII column (mAb, 2.4G2). The adsorbed supernatants were unable to induce IgG PFC responses but could induce IgA responses (Table 1). Importantly, material eluted from the anti-Fc γ RII column could augment IgG responses when recombined with its original adsorbed T_h sup. Similar results were seen when the T_h sup were adsorbed against an IgG column and the column eluate was recombined (Table 1) indicating that the factor that adsorbed to the anti-Fc γ RII column could induce IgG PFC as well as the IgGBF.

In parallel experiments, the T_h sup were adsorbed against an anti-Fc α R column, to determine whether the IgABF were structurally related to Fc α R. The resulting filtrates were devoid of any activity to augment IgA PFC but could still induce IgG responses (Table 1). The IgA-inducing activity was restored when the eluates from the anti-Fc α R column were added back to the adsorbed T_h sup (Table 1). Similar data was obtained when the co-culture supernatants were adsorbed against a monoclonal IgA column and the column eluates were added back to the adsorbed T_h sup (Table 1). Thus, material that was recognized by anti-Fc α R antibodies could induce IgA PFC as well as the IgABF.

Table 1. Antibodies to Fc γ RII and Fc α R recognize factors which enhance IgG and IgA PFC responses.

Treatment of T _h sup ^{a)}		SRBC-specific PFC ^{b)} no. per culture (\pm SEM) ^{c)}	
Adsorption	Eluate Added	IgG	IgA
Untreated	-	460(62)	610(86)
IgG	-	22(22)	350(60)
IgG	IgG	560(75)	500(80)
aFc γ RII	-	58(40)	380(56)
aFc γ RII	aFc γ RII	460(88)	540(100)
Untreated	-	500(80)	430(50)
IgA	-	400(76)	10(10)
IgA	IgA	460(85)	560(100)
aFc α R	-	390(79)	4(4)
aFc α R	aFc α R	410(78)	520(110)

- a) T_h sup were adsorbed on an IgG, IgA, anti-Fc γ RII (aFc γ RII) or anti-Fc α R (aFc α R) column, the eluates were derived from each of those columns.
- b) Cultures with neither T_s nor T_h sup had 470(60) IgG and 270(28) IgA SRBC-specific PFC and cultures with only T_s had 8(8) IgG and 41(23) IgA SRBC-specific PFC.
- c) Data are mean (SEM) from 6 experiments.

Factors recognized by anti-Fc γ RII or anti-Fc α R can replace IgGBF and IgABF in augmenting antigen-specific IgG and IgA PFC

Since the data described above show that both anti-FcR antibodies recognized factors that could augment IgG or IgA responses, we examined these factors for their ability to functionally substitute for the IgGBF and IgABF. T_h sup were adsorbed against either the IgG, IgA, anti-Fc γ RII or anti-Fc α R column. The adsorbed supernatants were then reconstituted with one of the eluates from these columns. When the T_h sup was adsorbed against the anti-Fc γ RII column and it was reconstituted with the IgG column eluate, the IgG PFC responses were restored. The reciprocal reconstitution, using T_h sup adsorbed against the IgG column and recombined with eluate from the anti-Fc γ RII column, also resulted in enhanced IgG PFC (Table 2). Therefore, the IgGBF and the factor isolated from the anti-Fc γ RII column were functionally identical (i.e. able to replace each other to promote IgG PFC). Similarly, when the IgA and anti-Fc α R adsorbed T_h sup were reconstituted with the eluates from the anti-Fc α R and IgA-column, respectively, both of the reconstituted supernatants could induce IgA PFC responses (Table 2).

Anti-Fc γ RII and Anti-Fc α R Antibodies Recognize Two Independent Factors

It has been previously demonstrated that the IgGBF (isolated on an IgG-column) and IgABF (isolated on an IgA-column) are two separate molecules with independent activities (1,5). The data in table 1 suggest that the anti-Fc γ RII and anti-Fc α R antibodies recognized factors that act to promote either IgG or IgA PFC because the anti-Fc γ RII antibody does not

Table 2. Factors recognized by anti-Fc γ RII and anti-Fc α R antibodies can replace IgGBF and IgABF.

Treatment of T _h sup ^{a)}		SRBC-specific PFC ^{b)} no. per culture (\pm SEM) ^{c)}	
Adsorption	Eluate Added	IgG	IgA
Untreated	-	1330(150)	1240(290)
IgG	-	22(22)	1040(170)
IgG	aFc γ RII	1190(120)	870(140)
aFc γ RII	-	55(55)	820(83)
aFc γ RII	IgG	1340(240)	890(100)
Untreated	-	860(97)	660(95)
IgA	-	910(100)	8(8)
IgA	aFc α R	1140(170)	470(70)
aFc α R	-	600(180)	11(11)
aFc α R	IgA	1350(140)	640(68)

- a) T_h sup were adsorbed on an IgG, IgA, anti-Fc γ RII (aFc γ RII) or anti-Fc α R (aFc α R) column, the eluates were derived from each of those columns.
- b) Cultures with neither T_h nor T_h sup had 880(58) IgG and 680(58) IgA SRBC-specific PFC and cultures with only T_h had 22(22) IgG and 10(10) IgA SRBC-specific PFC.
- c) Data are mean (SD) from 3 replicate cultures in 1 experiment.

remove IgA PFC activity from T_h sup and anti-Fc α R antibodies do not remove IgG PFC activity from T_h sup. In order to prove that these factors were separate entities, T_h sup were sequentially adsorbed against the anti-Fc γ RII and anti-Fc α R columns. The resulting adsorbed supernatants lacked IgG and IgA inducing activity (Table 3). When those adsorbed supernatants were recombined with the eluates from the anti-Fc γ RII column, only IgG PFC responses were reconstituted. Similarly, IgA responses returned when the anti-FcR adsorbed supernatants were recombined with the eluates from the anti-Fc α R column. Both IgG and IgA responses were restored when both eluates were added to the adsorbed supernatants. Thus the two factors isolated on the anti-FcR columns acted independently to promote isotype-specific PFC responses.

Isotype-Specific Reconstitution of IgG and IgA PFC Responses with rFc γ R and Affinity-Purified Fc α R

To further evaluate whether Fc γ RII can actually substitute for IgGBF, we used recombinant Fc γ R (rFc γ RII) (27) to reconstitute IgG responses in T_h sup that were adsorbed against an anti-Fc γ RII column. When 10 pg, 100 pg or 500 pg of rFc γ RII were added to adsorbed T_h sup, strong IgG PFC responses resulted while IgA PFC and direct IgM PFC were not affected. The enhancing effect of the rFc γ RII was titratable in that 1 pg gave no increase in IgG PFC while 10 or 100 pg did. However, adsorbed T_h sup combined with 10 ng to 100 ng of rFc γ RII showed reduced IgM, IgG and IgA PFC, although the latter was not consistently inhibited (Table 4). This high dose isotype-non-specific inhibition by rFc γ RII is

Table 3. Anti-Fc γ RII and anti-Fc α R antibodies recognize two independent factors that promote isotype-specific PFC responses.

Treatment of T _h sup ^{a)}		SRBC-specific PFC ^{b)} no. per culture (\pm SEM) ^{c)}	
Adsorption	Eluate Added	IgG	IgA
Untreated	-	780(130)	500(65)
aFc γ RII+aFc α R	-	9(9)	8(8)
aFc γ RII+aFc α R	aFc α R	7(7)	280(40)
aFc γ RII+aFc α R	aFc γ RII	360(70)	7(7)
aFc γ RII+aFc α R	aFc γ RII+aFc α R	320(21)	270(40)

- a) T_h sup were adsorbed on an IgG, IgA, anti-Fc γ RII (aFc γ RII) or anti-Fc α R (aFc α R) columns, the eluates were derived from each of those columns.
- b) Cultures with neither T_h nor T_b sup had 420(54) IgG and 360(23) IgA SRBC-specific PFC and cultures with only T_h had 11(11) IgG and 11(11) IgA SRBC-specific PFC.
- c) Data are mean (SEM) of 3 experiments.

Table 4. rFcγRII reconstitute isotype-specific IgG PFC responses.

Treatment of T _h sup ^{a)}		SRBC-specific PFC ^{b)} no. per culture (±SEM) ^{c)}		
Adsorption	FcR Added	IgG	IgA	IgM
Untreated	-	860(100)	660(100)	1600(280)
aFcγRII	-	39(39)	500(55)	1400(280)
aFcγRII	aFcγRII	460(87)	460(87)	1450(210)
aFcγRII	1 pg rFcγRII	7(7)	600(130)	1500(130)
aFcγRII	10 pg rFcγRII	810(150)	530(95)	1480(200)
aFcγRII	100 pg rFcγRII	1030(140)	550(44)	1810(360)
aFcγRII	500 pg rFcγRII	1300(350)	420(62)	1760(330)
aFcγRII	10 ng rFcγRII	49(28)	230(54)	160(80)
aFcγRII	100 ng rFcγRII	66(38)	460(33)	22(22)
aFcαR	100 pg rFcγRII	800(140)	12(12)	590(89)

- a) T_h sup were adsorbed on an anti-FcγRII (aFcγRII) or anti-FcαR (aFcαR) column, the eluates were derived from each of those columns.
- b) Cultures with neither T_h nor T_h sup had 760(116) IgG, 440(63) IgA and 1540(280) IgM SRBC-specific PFC and cultures with only T_h had 7(7) IgG, 18(18) IgA and 1790(310) IgM SRBC-specific PFC.
- c) Data are mean (SEM) from 5 experiments.

similar to that described by Varin *et al* (29). In addition, 100 pg of the rFcγR could not reconstitute IgA PFC responses in supernatants which had been adsorbed on the anti-FcαR column, indicating that rFcγR acted in an isotype-specific manner.

Similar results were obtained when affinity-purified FcαR (16,20) were used to functionally substitute for the IgABF (Table 5). T_h sup adsorbed against either an IgA-column or an anti-FcαR-column and combined with a 1:10 dilution of affinity purified FcαR could augment IgA PFC responses. The FcαR could not reconstitute the IgG PFC responses when combined with T_h sup that had been adsorbed against anti-FcγRII indicating that the FcαR was acting in an isotype-specific manner. There was insufficient FcαR available to try extremely concentrated dosages, in an attempt to determine if a high dose would result in isotype non-specific suppression.

The IgGBF Generated in the T_h sup contain soluble FcγR

To further evaluate whether the IgGBF were soluble FcγR, T_h sup were adsorbed against anti-FcγRII or IgG columns. The eluted proteins were concentrated and blotted onto nitrocellulose paper. FcγRII proteins were then detected with mAb 2.4G2 and the alkaline phosphatase detection method. FcγRII protein was detected in blotted serial dilutions of untreated T_h sup, eluates from the anti-FcγRII column and eluates from the IgG column (Fig. 1). Eluates from the IgA columns did not contain protein recognized by anti-FcγRII mAb in this assay (not shown). Densitometry of the bands and comparison to rFcγR blotted at the same time indicated that approximately equivalent amounts of FcγRII protein were eluted from the IgG and anti-FcγRII columns.

Table 5. Affinity-purified Fc α R reconstitute isotype-specific IgA PFC responses.

Treatment of T _h sup ^{a)}		SRBC-specific PFC ^{b)} no. per culture (\pm SEM) ^{c)}	
Adsorption	Eluate Added	IgG	IgA
Untreated	-	630(110)	600(80)
IgA	-	1200(220)	30(30)
IgA	1:10 Fc α R	470(89)	660(100)
aFc α R	-	1140(170)	11(11)
aFc α R	1:10 Fc α R	1070(140)	660(50)
aFc γ RII	1:10 Fc α R	8(8)	460(76)

- a) T_h sup were adsorbed on an anti-Fc α R (aFc α R) column, the eluates were derived from that column.
- b) Cultures with neither T_h nor T_h sup had 880(58) IgG and 680(58) IgA SRBC-specific PFC and cultures with only T_h had 22(22) IgG and 10(10) IgA SRBC-specific PFC.
- c) Data are mean (SEM) from 3 experiments.

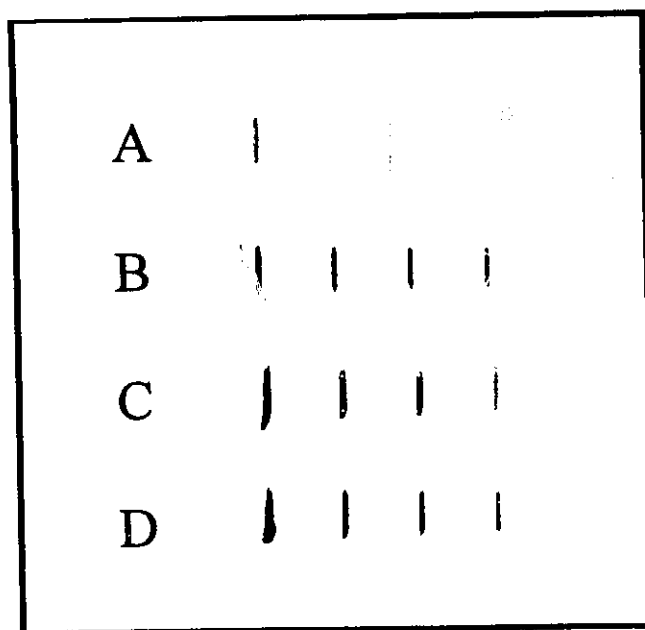


Figure 1 Detection of soluble FcγR in eluates from IgG and anti-FcγRII columns by protein slot-blot techniques.

Four fold serial dilutions (concentrated by a factor of 80 to 1.25, left to right) of untreated T_h sup (lane B), eluate from the anti-FcγR column (lane C) and eluate from the IgG-column (lane D) were blotted. rFcγR was slot-blotted at 1 μg, 1.0 ng, 0.5 ng and 0 ng (left to right) in lane A.

DISCUSSION

Previously we have reported that CD4⁺ T cells activated *in vitro* with either antigen-pulsed macrophage or IL-1 would generate IgG and IgA BF (1,5). These IgBF act to augment IgG or IgA PFC responses *in vitro* in the presence of T_h cells (1,5) and others have shown them to augment PFC responses *in vivo* (21). In this present study we have shown that IgGBF and IgABF generated *in vitro* by CD4⁺ T cells are functionally and serologically indistinguishable from FcR and that purified FcR can substitute functionally for the IgBF in the regulation of isotype-specific antibody responses *in vitro*.

The IgGBF share antigenic determinants with the FcγR because when adsorbed against the anti-FcγRII mAb, 2.4G2, the T_h sup could not augment IgG responses. Secondly, the eluates from the anti-FcγRII column could be used interchangeably with the eluates from the IgG-column to restore IgG PFC responses. Furthermore, slot-blot detection methods identified FcγRII proteins in eluates from both IgG and anti-FcγRII columns. These data demonstrate that the IgGBF, generated in the murine T_h sup, contains at least the extracellular domain of the FcγRII.

In similar experiments, T_h sup that were adsorbed against anti-FcαR antibodies could not augment IgA PFC. Further, the eluted material from the anti-FcαR column could be used interchangeably with the eluate from the IgA-column to restore IgA PFC when combined with T_h sup adsorbed against IgA. Although we could not, for technical reasons, identify FcαR in the slot-blot analysis of eluates from IgA or anti-FcαR columns, the supernatant adsorption data indicates that the IgABF contain components with serological identity with FcαR.

We next determined that purified soluble FcR could functionally substitute for the IgGBF. T_h sup depleted of IgGBF could support IgG PFC if rFcγRII was added. Similarly, purified FcαR reconstituted IgA PFC activity in T_h sup adsorbed against IgA. In the case of rFcγRII, we found that 10 pg to 500 pg of recombinant FcγR could functionally substitute for the IgGBF. The functional substitution of FcγRII for IgGBF and FcαR for IgABF means that FcR are sufficient as IgGBF. Taken together with the serological data, we conclude that the IgGBF and the IgABF in the T_h sup are in fact FcγRII and FcαR respectively.

The activity of the soluble FcR could be mediated via several mechanisms. The FcR could act directly upon T_h cells to prevent the suppression of isotype-specific antibody responses. In our system, however, this mode of activity is unlikely, since previous observations have demonstrated that the T_h sup have no direct effect upon T_h cell activity (31). The FcR may act on T_h that would alter isotype-specific responses indirectly. For instance, FcR activated T_h may interact with the B cells to promote isotype-switching (eg. cytokines) or somehow make the B cells resistant to T_h cell activity. Moreover, it is also possible that at low concentrations, such as those used in our study (10 pg/ml to 500 pg/ml), the FcR act directly upon the B cells to induce isotype-specific antibody responses.

It has been shown by others that FcR⁺ CD8⁺ T cells regulate Ig production through the generation of soluble FcR that act directly upon B cells to inhibit Ig production (15,17,32). We also observed that 10 ng/ml to 100 ng/ml of rFcγR would suppress IgG, IgM and IgA PFC responses. Our results are consistent with published data in which rFcγRII (2 ng/ml to 20 μg/ml) could inhibit PFC production *in vitro* (29). That work and other published data

indicate an antigen-non-specific suppression of B cell Ig production by soluble FcR (15,17,29,32), whereas our experiments involve the regulation of antigen-specific T_H cells that inhibit Ig production. We postulate that low concentrations of FcR provide isotype-specific augmentation of PFC responses that are otherwise suppressed by antigen-specific T_H cells. Within our model, FcR produced by the interaction of macrophages and CD4⁺ T cells, during the initial stages of antigen-specific response, limits the activity of antigen-specific T_H cells in an isotype specific manner.

This *in vitro* model may provide some understanding of Ig isotype regulation at mucosal surfaces. Gut associated lymphoid tissues are characterized by predominant IgA responses (2-4) and previous work has demonstrated that Peyer's patch CD4⁺ T cell hybridomas can generate soluble IgABF and express surface FcαR (1,30). The data shown here predict that Peyer's patch CD4⁺ T cells could interact with macrophage *in vivo* and release FcαR, thereby selectively increasing IgA production by Peyer's patch B cells, in the face of antigen-specific T_H cells. At the same time IgG response would still be suppressed. This fits with an interesting observation concerning the phenomenon of oral tolerance in which IgA antibody is produced while IgG antibody is suppressed (33,34).

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Chapter Four

Fc γ RIIB1 expression on CD4⁺ T cells

The following article entitled "Fc γ RIIB1 is expressed by normal alloreactive CD4⁺ T cells as an early activation marker" has been submitted to the Journal of Immunology. This study examines Fc γ RIIB1 expression on alloreactive CD4⁺ T cells and addresses the kinetics and some of the cellular requirements necessary for the induction of expression.

This work presented in this study was performed by the author of the thesis with the help of Barb Bagnerol, a clinical lab technician, with the FACS-sorting. This paper was written by the author of this thesis. Dr. Snider supervised this study.

TITLE:

FcγRIIB1 is expressed by normal alloreactive CD4⁺ T cells as an early activation marker.¹

RUNNING TITLE:

FcγRIIB1 is an early activation marker for CD4⁺ T cells.

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Footnotes

1. This work is supported by the Medical Research Council of Canada.
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ABSTRACT

We present evidence that FcγRIIB1 can be expressed by normal peripheral CD4⁺ T cells after MHC class II-restricted alloantigen presentation. Both splenic CD8⁺ and CD4⁺ T cells from B10.D2 mice, when alloactivated by B10.A spleen cells or a B10.A derived B lymphoma cell, expressed comparable levels of surface FcγRII/III, as defined by mAb 2.4G2. However, while nearly all alloreactive CD8⁺ cells appeared to express FcγRII/III, only a fraction of the CD4⁺ T cells had surface FcγRII/III. Alloactivation of purified CD4⁺ T cells, resulted in a 10 to 20 fold increase in the total number of CD4⁺ T cells that expressed FcγRII/III, within 24 hours, and prior to DNA synthesis. The expression of surface FcγRII/III on CD4⁺ T cells could be blocked by anti-MHC class II mAb, indicating that recognition of MHC class II was required. RT-PCR identified mRNA for the FcγRIIB1 isoform in the activated CD4⁺ T cells. In addition, both surface expression and mRNA for FcγRIIB1 were induced by 24 hours on FACS-sorted CD4⁺ T cells, that had no surface expression prior to culture. Thus, the FcγRIIB1 expression was *de novo*, and was not an expansion of preexisting CD4⁺ T cells that expressed FcγRIIB1. Finally, anti-CD3 treatment of CD4⁺ T cells did not induce FcγRIIB1 surface expression, indicating that TcR engagement alone, even in the presence of IL-2, was not sufficient for FcγRIIB1 expression. These studies clearly demonstrate that normal mature CD4⁺ T cells express FcγRIIB1 mRNA and surface FcγRII/III protein soon after antigenic stimulation.

Introduction

Fc receptors (FcR) for each of the five immunoglobulin (Ig) isotypes have been identified on a variety of haematopoietic cells (1). Initial studies that investigated T cell FcR expression, demonstrated that FcR⁺ CD8⁺ T cells can be generated *in vivo* through the adoptive transfer of B cell plasmacytomas or hybridomas (2-5). Fc γ R are among those FcR that have been identified on murine CD8⁺ T cells (3). Furthermore, murine CD8⁺ T cells ($\alpha\beta$ -TcR expressing clones) only express the Fc γ RIIB1 isoform (6). Other CD8⁺ T cells that express $\gamma\delta$ -TcR, including intestinal IEL, can be induced to express Fc γ RIII following activation with anti-CD3 (7,8). Functionally, both FcR⁺ CD8⁺ T cells (9,10) and T cell lines (11) have been shown to down-regulate B cell Ig production, *in vivo* (4,9,12) and *in vitro* (10,11), through the generation of soluble FcR (11,13,14).

Considerably less data is available on the ability of mature peripheral CD4⁺ T cells to express Fc γ R. Lynch, Sandor, and colleagues have reported that some CD4⁺ T cell clones, can express surface Fc γ RII or Fc γ RIII, after MHC class II-restricted antigen-presentation (15). In that single study, expression was noted only on long-term cultured, and repeatedly stimulated Th2 clones. The Fc γ R isoform produced by those CD4⁺ T clones was not clearly identified. A recent report has shown that early T cell precursors in the thymus express multiple forms of Fc γ R (both type II and III) and the engagement of these FcR can promote thymocyte differentiation (16,17).

We set out to better define the nature of Fc γ R expression on mature peripheral CD4⁺ T cells, comparing that expression to CD8⁺ T cells, using allogeneic stimulation *in vitro*.

Based on FACS analysis and RT-PCR, we show that splenic CD4⁺ T cells can be activated to express FcγRIIB1 *de novo*. This expression is dependent on recognition of MHC class II in the alloreaction culture, but CD4⁺ T cells do not express FcγRIIB1 simply by CD3 ligation. Further, the induction of FcγRIIB1 expression on CD4⁺ T cells occurs within 24 hours of activation, indicating that FcγRIIB1 is an early activation marker of mature CD4⁺ T cells.

Materials and Methods

Mice.

Female B10.A and B10.D2N mice (Jackson Laboratories, Bar Harbour, MA) were used between 8-10 weeks of age and housed under conventional conditions at McMaster University.

Antibodies and Cell Lines

The monoclonal antibodies purchased for FACS analysis were 2.4G2 (18) anti-Fc γ RII/III-PE, (Pharmagen, San Diego, CA), goat-anti-mouse IgM-PE (Jackson ImmunoResearch, West Grove, PA), (goat-anti-mouse IgM-biotin, Southern Biotechnology, Birmingham, AL). Others were isolated on either protein-G or protein-A columns from tissue-culture supernatants or ascites and labelled with FITC or biotin in our lab using standard techniques (19). These include 6B2 (20), anti-B220, 145-2C11 (21), anti-CD3 (CRL 1975, ATCC, Rockville, MD), GK1.5 (22), anti-CD4 (TIB 207, ATCC) and 53-6.72 (23), anti-CD8 (TIB 105, ATCC). The mAb YTS 169.4, anti-CD8 (Cedarlane, Hornsby, Ontario), J11d.2 (24), anti-heat stable antigen (HSA), (TIB 183, ATCC) and M5/114.15.2 (25), anti-I-A^{b4q} and anti-I-E^{dk}, (TIB 120, ATCC) were used to isolate CD4⁺ T cells. 10-2.16 (26), anti-I-A^k, (TIB 93, ATCC) and M5/114.15.2 were used to block MHC class II-restricted alloactivation. The CH12.LX B cell line that secretes IgG2b was generously provided by Dr. Geoffrey Houghton, UNC (27). All cells were cultured in RPMI 1640

supplemented with penicillin, streptomycin, L-glutamine, sodium pyruvate, HEPES, 2-ME and 10% FCS (10%FCS-RPMI).

Isolation of CD4⁺ T cells

Splenic CD4⁺ T cells were isolated by incubating nylon wool nonadherent B10.D2 spleen cells at 10×10^6 per ml of HBSS containing 5 μ g of anti-I-A^d, a 1:5 dilution of anti-HSA supernatant and a 1:300 dilution of anti-CD8 (YTS 169.4), for 45 min at 4° C. The cells were subsequently incubated with a 1:10 dilution of rabbit complement (Cedarlane) for 1 hour at 37° C. The splenic CD4⁺ T cells were then analyzed for viability and B220, IgM, CD3, CD4, CD8 and Fc γ RII/III surface expression by FACS analysis. The purified CD4⁺ T cells were 80 \pm 5% CD4⁺, 79 \pm 4% CD3⁺, 3 \pm 3% IgM⁺, 3 \pm 2% B220⁺, 0.2 \pm 0.2% CD8⁺ and 96 \pm 3% viable prior to coculture. After allogeneic coculture with CH12.LX B cells for 72 hours the cells were 96 \pm 4% CD4⁺, 96 \pm 2% CD3⁺, 1 \pm 1% IgM⁺, 1 \pm 1% B220⁺, and 94 \pm 5% viable (data from 10 experiments).

Activation of CD4⁺ T cells to Induce Fc γ RII/III Surface Expression

Purified B10.D2 CD4⁺ T cells (10×10^6) were cultured with 5×10^6 irradiated allogeneic B10.A splenocytes (2000 rads) or 5×10^6 irradiated CH12.LX B cells (5000 rads) for 24 to 72 hours, in 6 well plates (Corning Inc., Corning, NY) containing 10%FCS-RPMI. The ratio of responder CD4⁺ T cells to stimulators was determined for optimal CD4⁺ T cell proliferation (see below). In some experiments 2×10^6 purified CD4⁺ T cells were activated,

for 24 to 72 hours, with rhIL-2 (20 U/ml) (Boehringer Mannheim, Laval, Quebec) in 6 well plates (Corning) coated with anti-CD3 (145-2C11) at 33 $\mu\text{g/ml}$. For anti-MHC class II blocking, a mixture of anti-MHC class II mAb (M5 and 10-2.16) at a final concentration of 7.5 $\mu\text{g/ml}$ was added to some cocultures.

Proliferation Cultures and Assay

CD4⁺ T cell proliferative responses were determined by [³H]-thymidine incorporation (1.0 $\mu\text{Ci/well}$) (NEN, Boston, MA). Various numbers (8×10^5 to 6×10^3) of CD4⁺ T cells were incubated with (8×10^5 to 1×10^5) B10.A splenocytes or CH12.LX B cells irradiated with 2000 or 5000 rads, respectively. These cultures were conducted in 96 well flat bottom plates (Corning) in 10%FCS-RPMI. Cultures were harvested using a PHDTM cell harvester (Cambridge Technologies, Inc., Cambridge, MA). Samples were counted on a Beckman LS-5801 beta counter (Beckman Instruments, Mississauga, Ontario).

Flow Cytometry Analysis and FACS-Sorting of CD4⁺ T cells

CD4⁺ T cells were suspended in 50 μl of PBS/0.2% BSA/0.1% NaN₃ (PBS/BSA) at a concentration of 1×10^7 cells/ml, with 75 μl of mixtures of up to 3 labelled diluted mAb (optimal titrations), at 4° C for 30 minutes. The cells were subsequently washed and then incubated in 50 μl PBS/BSA with 10 μl of strepavidin-PerCP (Becton Dickinson, San Jose, CA) at 4° C for 15 minutes, in order to detect the biotinylated mAb. Cells were washed again, suspended in 500 μl of PBS/BSA and analyzed on a FACScan (Becton Dickinson). For

analysis of Fc γ RII/III expression on CD4⁺ and CD8⁺ T cells 5×10^4 events were collected. To identify Fc γ RII/III⁺ CD4⁺ T cells, cells were stained with anti-CD4-FITC, anti-Fc γ RII/III-PE and anti-B220-biotin or anti-IgM-biotin. Fc γ RII/III⁺ CD8⁺ T cells were identified in the same manner but using anti-CD8-FITC. CD4⁺ and CD8⁺ T cell viability was determined using propidium iodide staining with anti-CD4- or and anti-CD8-FITC. Compensation adjustments were done on sets of single-stained cells. Flow cytometry data was analyzed utilizing PC-LYSYS software (Becton Dickinson).

Fc γ RII/III⁺ CD4⁺ T cells were purified from the initial CD4⁺ T cell preparation using FACS-sorting. Briefly, freshly isolated splenic CD4⁺ T cells were stained with anti-CD4-FITC and anti-Fc γ RII/III-PE. The cells were washed, suspended to 5×10^6 cells/ml in 10%FCS-RPMI and the Fc γ RII/III⁺ CD4⁺ cells were purified utilizing a FACStar^{Plus}, on the basis of forward scatter and exclusion of Fc γ RII/III-PE staining (Becton Dickinson). The sorted cells were subsequently stained with anti-B220-biotin and streptavidin-PerCP (Becton Dickinson) to determine purity.

In some experiments, CD4⁺ T cells were sorted after culture with the CH12.LX B cells for 24 or 72 hours. Harvested cells were stained with anti-CD4-FITC, washed, suspended to 5×10^6 cells/ml in 10%FCS-RPMI and the CD4⁺ T cells were purified by positive sorting. An aliquot of the sorted cells were stained with anti-Fc γ RII/III-PE and anti-B220-biotin or anti-IgM-biotin for 30 minutes at 4° C, washed and then incubated with streptavidin-PerCP (Becton Dickinson) for 15 minutes at 4° C. These cells were washed and then analyzed to determine purity of the CD4⁺ T cells.

Calculation of Total Number of CD4⁺ or CD8⁺ FcγRII/III⁺ T cells

Flow cytometry data, obtained from analysis of triple staining with anti-CD4-FITC, anti-FcγRII/III-PE and anti-B220- or anti-IgM-biotin-streptavidin-PerCP, was used to quantitate FcγRII/III⁺ CD4⁺ T cells. Lymphocytes were gated by forward and side scatter parameters. A second gate, defined all positive CD4-FITC staining cells. Combining the two gates allowed analysis of CD4⁺ T cells for expression of FcγRII/III and B220 (or IgM) in the second and third colours. The total number of CD4⁺ FcγRII/III⁺ lymphocytes in the analyzed sample and the small number of contaminating B cells (defined as B220⁺ or IgM⁺ and FcγRII/III⁺) were determined by two colour analysis. The number of contaminating B cells were subtracted from the number of FcγRII/III⁺ CD4⁺ lymphocytes yielding the actual number of true FcγRII/III⁺ CD4⁺ T cells within the analyzed sample. The percent of FcγRII/III⁺ CD4⁺ T cells in the analyzed sample was determined by dividing the total number of FcγRII/III⁺ CD4⁺ T cells by the total number of lymphocytes in the scatter gate. A total viable lymphocyte count was made based on trypan blue exclusion and counting on a haemocytometer. The total number of FcγRII/III⁺ CD4⁺ T cells was then calculated by multiplying the total number of viable lymphocytes harvested from the coculture times the percent of FcγRII/III⁺ CD4⁺ T cells. A similar calculation procedure was also used to determine the total number of FcγRII/III⁺ CD8⁺ T cells in the cocultures, when CD8⁺ T cells were identified with anti-CD8-FITC.

Detection of Fc γ RIIB1 mRNA

RNA was isolated from CD4⁺ T cells using phenol/chloroform extraction as previously described (28). After washing in PBS, 2.5×10^6 CD4⁺ T cells were lysed in 0.5 ml of cold buffer containing 4 M GITC, 25 mM sodium citrate, 0.1 M 2-ME and 0.5% sarcosine, pH 7.4. After incubation on ice for 5 minutes, 50 μ l of 2 M sodium acetate, pH 4.0, 100 μ l of chloroform-isoamyl alcohol (49:1) and 0.5 ml of Tris-buffer saturated phenol was added. The lysate was centrifuged at 5000 g for 15 min at 4 $^{\circ}$ C, the aqueous phase was collected, mixed with an equal volume of isopropanol and then incubated at -20 $^{\circ}$ C for 18 hour. The sample was then centrifuged at 5000 g for 10 minutes at 4 $^{\circ}$, the supernatant was removed and the RNA pellet was washed in 75% ethanol with 0.3 M sodium acetate at -20 $^{\circ}$ C. After an 18 hour incubation, the RNA was pelleted by centrifugation at 5000 g for 10 minutes at 4 $^{\circ}$ C. The RNA pellet was dried and then dissolved in 200 μ l of water.

For RT-PCR, 1 μ g of RNA was added to a RT reaction mixture containing 50 mM Tris-HCl, 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, 2.5 μ M oligo dT₍₁₈₎, 0.5 mM each of dGTP, dTTP, dCTP, dATP and 15 U RNase inhibitor (Pharmacia, Uppsala, Sweden). 200 U of MMLV reverse transcriptase (GIBCO BRL, Burlington, Ontario) was added and the reaction mixture was incubated for 10 minutes at 20 $^{\circ}$ C and then at 37 $^{\circ}$ C for 70 minutes. The reaction was stopped by heating at 95 $^{\circ}$ C for 5 minutes and then placed at 4 $^{\circ}$ C. The cDNA synthesized by RT was then amplified by PCR.

The following primer pairs were used for the PCR amplification for β -actin, 5' primer - CTCTTTGATGTCACGCACGATTTC, 3' primer - GTGGGCCGCTCTAGGCACCAA and

for FcγRIIB1; 5' primer - AAGCAGGTTCCAGCTCTC (cDNA sequence bp 1054 to bp 1072, exon 7 and 8) and 3' primer - GCTGCTGCTTGTTGGCTC (cDNA sequence bp 1184 to bp 1202, exon 8) (29). The predicted sizes of the amplified DNA are 540 bp for β-actin and 148 bp for FcγRIIB1. 80 µl of a PCR mixture containing 1 M MgCl₂, 10 mM Tris-HCl, 50 mM KCl, 1 µM of each primer and 5 U of Taq (Boehringer Mannheim) was added to the 20 µl of RT reaction. The samples were overlaid with 75 µl of mineral oil and then amplified in a DNA thermal cycler (Perkin Elemer Cetus, Emeryville, CA) for 35 cycles: denaturation at 95°C for 1 minute, primer annealing at 53° C for 2 minutes and a final extension was performed at 53° C for 7 minutes. The amplified samples were electrophoresed on a 2% agarose gel containing 0.5 µg/ml ethidium bromide and after electrophoresis, the agarose gel was photographed under UV light.

Results

Alloactivation of splenic lymphocytes, *in vitro*, induces an increase in the total number of CD4⁺ and CD8⁺ T cells that express FcγRII/III.

We investigated whether activation of splenic CD8⁺ and CD4⁺ T cells with irradiated allogeneic spleen cells could induce FcγRII or FcγRIII surface expression, using the mAb 2.4G2 that recognizes either type (18,30). B10.D2 spleen cells were cultured with irradiated B10.A spleen cells for 72 hours, harvested and the CD4⁺ and CD8⁺ T cells were examined for FcγRII/III surface expression. Prior to culture, $2.4 \pm 0.3\%$ of CD8⁺ T cells and $6.1 \pm 0.2\%$ of CD4⁺ spleen T cells from the B10.D2 mouse expressed FcγRII/III. Interestingly, the number of FcγRII/III⁺ CD4⁺ T cells were 7 fold higher than FcγRII/III⁺ CD8⁺ T cells in the normal spleen (Table 1) and their ratio was not reflected in the normal CD4:CD8 ratio that was 2:1 (n=3). After culture almost all CD8⁺ T cells ($98 \pm 2\%$) expressed low levels of FcγRII/III, relative to normal B cell expression (Fig. 1b). A significant fraction ($16 \pm 3\%$) of the CD4⁺ T cells also expressed FcγRII/III after 72 hours of culture (Fig. 1a). The number of FcγRII/III⁺ CD8⁺ T cells increased 69 ± 15 fold (n=3), whereas the number of FcγRII/III⁺ CD4⁺ T cells increased 6 ± 0.9 fold (n=4). These results indicated that an expansion in the total number of CD8⁺ and CD4⁺ T cells that expressed FcγRII/III was induced by alloactivation. Moreover, the FcγRII/III⁺ CD8⁺ and FcγRII/III⁺ CD4⁺ T cells had comparable intensities of FcγRII/III staining indicating that both cells expressed similar amounts of surface FcγRII/III (Fig. 1a,b).

Others have described the generation of FcR⁺ CD8⁺ T cells by coculturing human PBL with allogeneic B cell lines (31). Therefore, we cocultured B10.D2 spleen cells with the

Table 1. Alloactivation of splenic lymphocytes stimulates an increase in the total number of FcγRII/III⁺ T cells.

	Stimulators ^b	Added or recovered viable cells per culture ^a ($\times 10^{-3}$)		Mean Increase (\pm SD)
		Pre-culture	Post-culture	
CD8 ⁺	B10.A spleen	9	690	69(15)
		13	680	
		8	630	
CD8 ⁺	CH12.LX	9	780	86(5)
		13	1030	
		8	730	
CD4 ⁺	B10.A spleen	63	310	6(0.9)
		54	260	
		85	560	
		67	420	
CD4 ⁺	CH12.LX	63	530	9(0.8)
		54	540	
		85	730	
		67	640	

- a) Data are the total number of viable CD8⁺ or CD4⁺ FcγRII/III⁺ T cells ($\times 10^{-3}$) added to or recovered per culture in 3 or 4 experiments. 10×10^6 total responder cells were added to each culture. 50×10^3 events were analyzed for each determination.
- b) B10.D2 splenocytes were harvested after coculture for 72 hours with irradiated B10.A splenocytes or CH12.LX B cells.

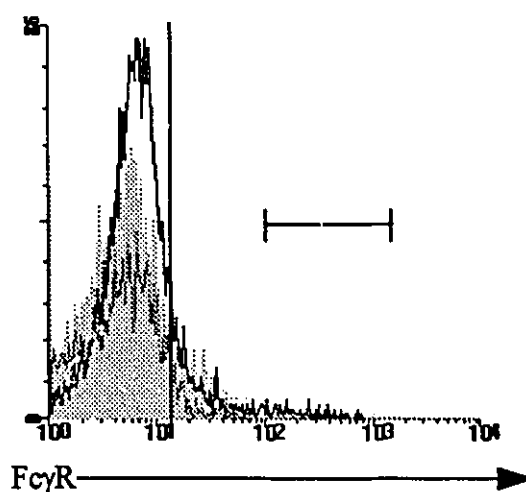
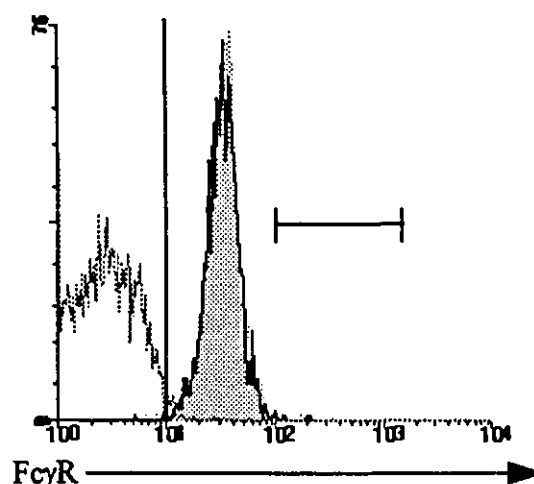
a) CD4⁺ T cellsb) CD8⁺ T cells

Figure 1 Alloactivation of splenic lymphocytes induces FcγRII/III expression on CD4⁺ and CD8⁺ T cells.

Data are from 1 representative experiment. CD4⁺ (a) and CD8⁺ (b) T cells were stained with anti-FcγRII/III-PE prior to coculture (dotted line), after 72 hours (solid line) of coculture with B10.A spleen cells, 12% of CD4⁺ and 98% of CD8⁺ were FcγRII/III⁺. Surface expression by CD8⁺ and CD4⁺ T cells taken at 72 hours from separate cocultures, using CH12.LX B cells, are stained in solid grey (15% CD4⁺ and 99% CD8⁺ were FcγRII/III⁺). All B220⁺ cells were excluded from the analysis of CD4⁺ and CD8⁺ T cells. Vertical line represents autofluorescence from 95% of all non-stained CD4⁺ or CD8⁺ T cells. Horizontal bar includes 95% of all B10.D2 splenic B cells stained with FcγRII/III-PE. CD4⁺ and CD8⁺ T cells were 96±3% viable by PI staining.

CH12.LX B cell lymphoma to determine if Fc γ RII/III could be induced on alloreactive CD4⁺ T cells. The CH12.LX cell line was derived from B10.A mice (27) and is therefore comparable to the alloactivation by B10.A spleen. Initial experiments determined whether the CH12.LX B cells could stimulate the B10.D2 splenocytes to proliferate. Data from these experiments demonstrated that appropriate numbers of irradiated CH12.LX B cells could stimulate proliferation of B10.D2 splenocytes that was comparable to B10.A allostimulation (Fig.2). Once MLR conditions were established, B10.D2 spleen cells were cocultured with CH12.LX B cells for 72 hours, harvested and the CD8⁺ and CD4⁺ T cells were examined for Fc γ RII/III expression. An 86 ± 5 (n=3) fold increase in the total number of Fc γ RII/III⁺ CD8⁺ T cells was observed within 72 hours of activation (Table 1). Furthermore, a 9.2 ± 0.8 (n=4) fold increase in the total number of CD4⁺ Fc γ RII/III⁺ T cells was also observed within 72 hours of activation (Table 1). Thus CH12.LX B cells could effectively substitute for B10.A splenic APC in the induction of Fc γ RII/III expression on CD4⁺ T cells from B10.D2 spleen.

Alloactivation of CD4⁺ T cells induces Fc γ RII/III expression within 24 hours.

The preceding experiments showed that induction of Fc γ RII/III on CD4⁺ T cells within the whole responder splenocyte population was moderate with only 10 to 16% of CD4⁺ T cells showing Fc γ RII/III expression by 72 hours. In addition, we wished to determine how quickly Fc γ RII/III was expressed by CD4⁺ T cells and if it was related to a proliferative expansion of alloreactive cells. We therefore purified splenic CD4⁺ T cells and examined the kinetics of Fc γ RII/III expression during their alloactivation by CH12.LX B

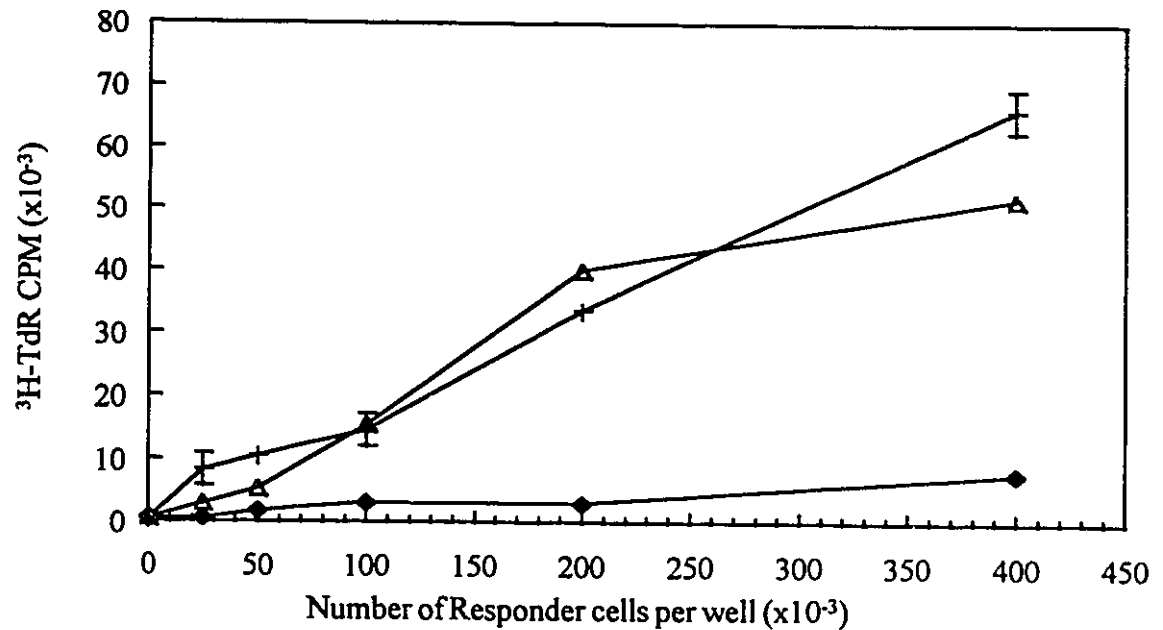


Figure 2 CH12.LX B cells were compared to B10.A spleen cells for their ability to stimulate B10.D2 T cell proliferation.

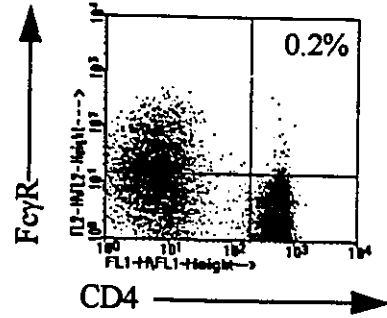
Various numbers (x-axis) of B10.D2 splenocytes were cultured alone (◆) or with either 200×10^3 B10.A splenocytes (+) or 200×10^3 CH12.LX B cells (Δ) for 96 hours. Cultures were pulsed with [³H]-thymidine 24 hours prior to harvesting. Data are mean CPM values, \pm SD, from one representative experiment.

cells. The viable cells added at the start of the coculture were $80 \pm 5\%$ CD4⁺, $3 \pm 3\%$ B220⁺, $3 \pm 3\%$ IgM⁺ and the remaining cells were null-staining. After 72 hours of coculture, the viable harvested cells were $96 \pm 4\%$ CD4⁺, $1 \pm 1\%$ B220⁺ and $1 \pm 1\%$ IgM⁺. FcγRII/III⁺ CD4⁺ T cells were detected within the activated populations (Fig. 3b,c,d) and a 15 fold increase in the total number of FcγRII/III⁺ CD4⁺ T cells was observed by 24 hours, a 32 fold increase by 48 hours and a 44 fold increase after 72 hours (Fig. 3, Table 2). This numeric increase corresponded to a relative increase of FcγRII/III⁺ CD4⁺ T cells from $\leq 1\%$ preculture to $10 \pm 1\%$ (24 h), $16 \pm 5\%$ (48 h) and $26 \pm 5\%$ (72 h). These results indicated that alloactivation of CD4⁺ T cells could induce an expansion in the total number of FcγRII/III⁺ CD4⁺ T cells within 24 hours. When the proliferation kinetics of alloactivated CD4⁺ T cells were examined, no [³H]-thymidine uptake was apparent by 24 hours (Fig. 4). Thus, the expression of FcγRII/III on CD4⁺ T cells had occurred prior to any detectable DNA synthesis.

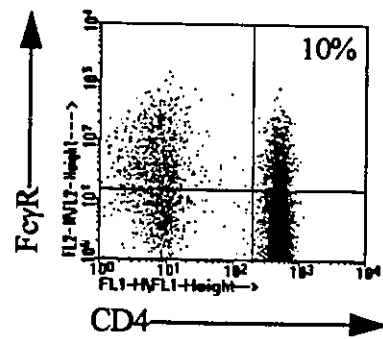
Alloactivation of CD4⁺ T cells induces *de novo* FcγRII/III expression.

We next investigated whether the FcγRII/III⁺ CD4⁺ T cells had resulted from an expansion of the small number of initial FcγRII/III⁺ CD4⁺ T cells, or if the FcγRII/III expression detected on the CD4⁺ T cells had occurred *de novo*. FcγRII/III⁺ CD4⁺ T cells, isolated from CD4⁺ T cell preparations by FACS-sorting (see Methods and Materials), were cocultured with CH12.LX B cells for 24 or 72 hours and then examined for FcγRII/III expression. The FACS-sorted cells were $95 \pm 3\%$ CD4⁺ and $0.10 \pm 0.06\%$ FcγRII/III⁺ CD4⁺ prior to alloactivation. After 24 hours of activation, there was on average an 83 fold increase

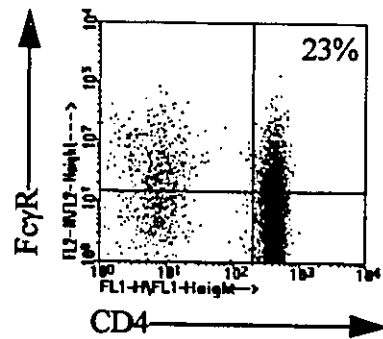
a) 0 Hours



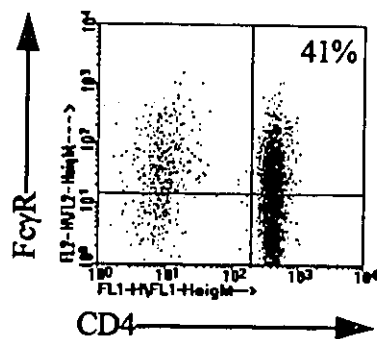
b) 24 Hours



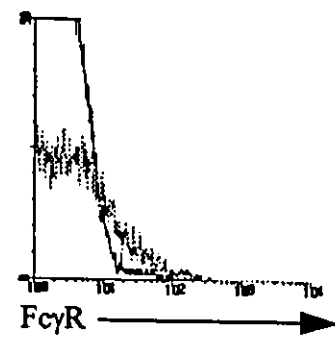
c) 48 Hours



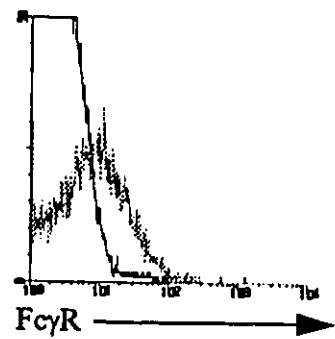
d) 72 Hours



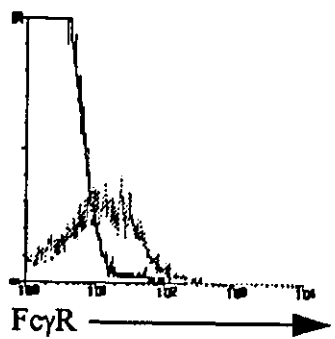
e) 24 Hours



f) 48 Hours



g) 72 Hours



Legend to Figure 3

Kinetics of FcγRII/III expression on purified B10.D2 CD4⁺ T cells during alloactivation. The data are from one representative experiment. The dotplots show FcγRII/III-PE (y-axis) and CD4-FITC (x-axis) prior to coculture (a), at 24 (b), 48 (c) or 72 hours (d) of culture. The histograms show FcγRII/III-PE staining on CD4⁺ T cells prior to (solid line) or after culture (dotted line) with CH12.LX B cells for 24 (e), 48 (f) or 72 hours (g). CD4⁺ T cells were 95±3% viable by PI staining.

Table 2. Alloactivation of CD4⁺ T cells induces FcγRII/III expression within 24 hours.

Time in culture ^b	Added or recovered viable cells per culture ^a (×10 ³)		Mean Increase (±SD)
	Pre-culture	Post-culture	
24	32	400	15(5)
	32	430	
	29	290	
	25	430	
	14	330	
48	32	990	32(5)
	32	1100	
72	14	630	44(7)
	29	1500	
	23	740	
	32	1400	
	25	1200	

a) Data are the total number of viable CD4⁺ FcγRII/III⁺ T cells (×10³) added to or recovered per culture in 5 experiments. 10×10⁶ total responder cells were added to each culture. 50×10³ events were analyzed for each determination.

b) Purified CD4⁺ T cells were cocultured with irradiated CH12.LX B cells.

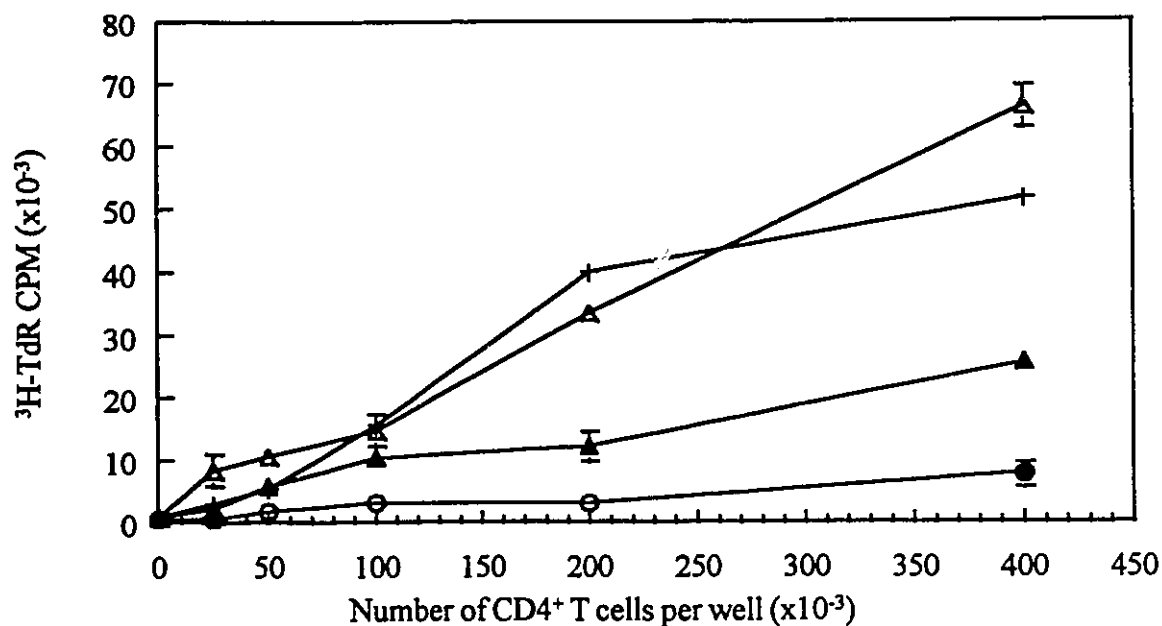


Figure 4 Proliferation kinetics of CD4⁺ T cells alloactivated with CH12.LX B cells.

Various numbers of B10.D2 splenocytes (x-axis) were incubated with irradiated CH12.LX B cells (200×10^3) for 24 to 96 hours. Cultures were pulsed with [³H]-thymidine at 24 (O), 48 (▲), 72 (Δ) or 96 hours (+) of coculture. Cultures of CD4⁺ T cells alone were pulsed at 72 hours (●). Data are mean CPM values, \pm SD, from one representative experiment.

Table 3. Alloactivation of FACS-sorted FcγRII/III⁺ CD4⁺ T cells induces *de novo* FcγRII/III expression.

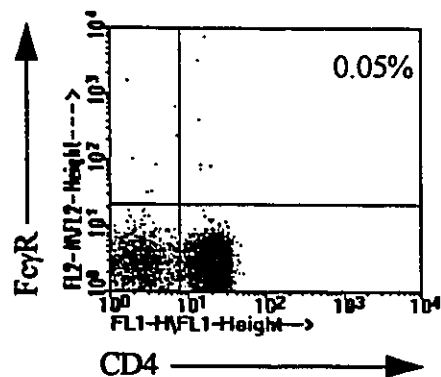
Time in culture ^a	Added or recovered viable cells per culture ^a (×10 ⁻³)		Mean Increase (±SD)
	Pre-culture	Post-culture	
24	2.4	180	83(8)
	7.1	640	
	8.0	670	
72	0.7	100	130(18)
	0.6	90	
	0.7	79	
	2.3	270	

- a) Data are the total number of viable CD4⁺ FcγRII/III⁺ T cells (×10⁻³) added to or recovered from each culture. 10×10⁶ total responder cells were added to each culture. Data are from 7 experiments. 50×10³ events were analyzed for each determination.
- b) FACS-sorted FcγRII/III⁺ CD4⁺ T cells were cocultured with irradiated CH12.LX B cells for 24 or 72 hours.

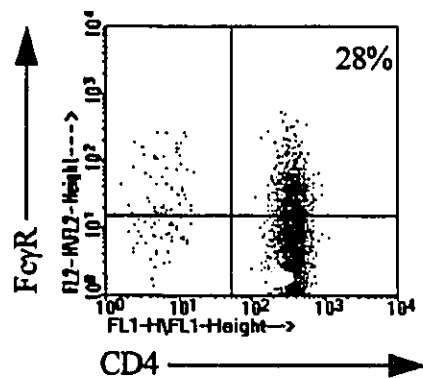
in the total number of Fc γ RII/III⁺ CD4⁺ T cells (Table 3). After 72 hours of activation, there was a 130 fold increase. Furthermore, 28 \pm 2% of the CD4⁺ T cells were Fc γ RII/III⁺ after 24 hours of culture and after 72 hours 33 \pm 2% were Fc γ RII/III⁺ (Fig.5).

Based on the known expression of Fc γ RIIB1 isoform on CD8⁺ T cells (6) and B cells (32), it seemed likely that the alloactivated CD4⁺ T cells would express this Fc γ RII isoform. We therefore examined the alloactivated CD4⁺ T cells for Fc γ RIIB1 mRNA using RT-PCR. FACS-sorted Fc γ RII/III⁻ CD4⁺ T cells were alloactivated with CH12.LX B cells for 24 or 72 hours. Since CH12.LX B cells express Fc γ RIIB1 mRNA (Fig. 6, lane 7), the cells harvested at the end of culture were FACS-sorted a second time to isolate only the CD4⁺ T cells and to remove any contaminating CH12.LX B cells. The level of contaminant of CH12.LX B cells in the final preparation of cells was reduced to \leq 0.01%, based on B220-PerCP staining. RNA was isolated from both pre- and post-culture populations of FACS-sorted CD4⁺ T cells. Fc γ RIIB1 mRNA could be detected in the RNA preparations isolated from the FACS-sorted CD4⁺ T cells that had been alloactivated for either 24 or 72 hours (Fig. 6, lanes 2-5), but not from Fc γ RII/III⁻ CD4⁺ T cells prior to culture (Fig. 6, lane 1). To evaluate whether the 0.01% contamination of CH12.LX B cells could have contributed significant Fc γ RIIB1 mRNA, RT-PCR was also performed on an RNA preparation isolated from FACS-sorted FcR⁻ CD4⁺ T cells that had been admixed with a 0.01% contaminant of CH12.LX B cells. Fc γ RIIB1 mRNA could not be detected in these RNA preparations (Fig. 6, lane 10). Thus, the RT-PCR product from the post-culture CD4⁺ T cells could not be explained by the 0.01% CH12.LX contamination. The RT-PCR results and the surface expression data (Fig. 5)

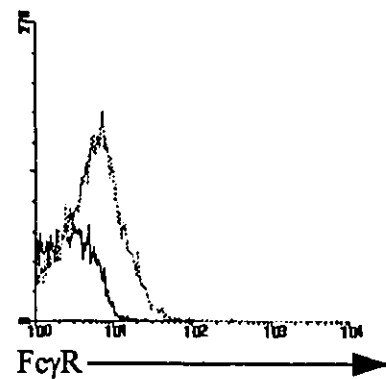
FACS-sorted FcR⁻
a) CD4⁺ T cells



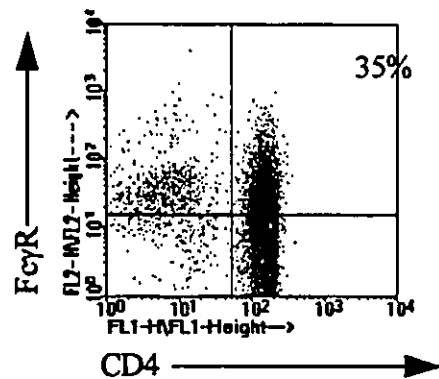
b) 24 Hours



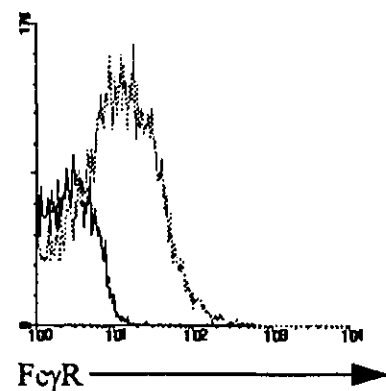
d) 24 Hours



c) 72 Hours



e) 72 Hours



Legend to Figure 5

Alloactivation of FACS-sorted Fc γ RII/III⁺ CD4⁺ T cells induces Fc γ RII/III expression.

The data are from one representative experiment. The dotplots show Fc γ RII/III-PE (y-axis) and CD4-FITC (x-axis) staining on FACS-sorted Fc γ RII/III⁺ CD4⁺ T cells prior to coculture (a), and at 24 (b) or 72 hours (c) of culture with CH12.LX B cells. The histograms show Fc γ RII/III-PE staining on the FACS-sorted Fc γ RII/III⁺ CD4⁺ T cells prior to (solid line) and after coculture (dotted line) for 24 (d) or 72 hours (e). CD4⁺ T cells were 95 \pm 2% viable by PI staining. 50 \times 10³ events were analyzed for each determination.

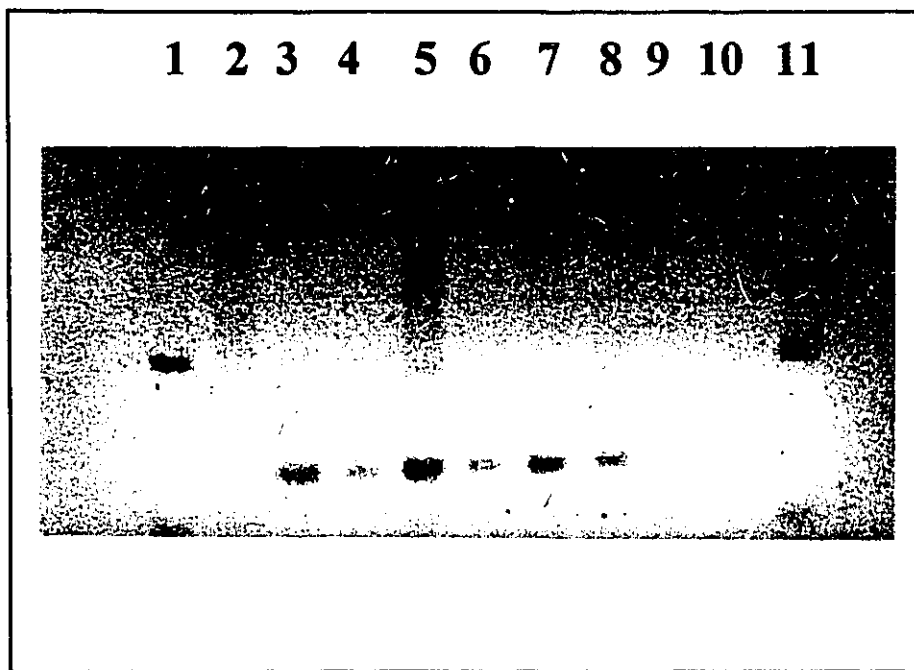


Figure 6 Alloactivation of FACS-sorted Fc γ R II/III CD4 $^{+}$ T cells induces *de novo* expression of Fc γ RIIB1 mRNA within 24 hours.

RT-PCR, to detect Fc γ RIIB1 mRNA, was performed on 1 μ g of RNA from FACS-sorted Fc γ R II/III CD4 $^{+}$ T cells prior to coculture (lane 2), 1 μ g or 0.1 μ g of RNA from CD4 $^{+}$ T cells that were purified by FACS-sorting after 24 (lanes 3,4) or 72 hours (lanes 5,6) of alloactivation. Controls include 1 μ g of RNA from CH12.LX B cells (lane 7), 1 μ g of B10.D2 spleen RNA (lane 8), 1 μ g of 3T3 fibroblast RNA (lane 9). Lane 10 shows RT-PCR reaction product from 1 μ g of RNA isolated from Fc γ R II/III CD4 $^{+}$ T cells that had been admixed with a 0.01% contamination of CH12.LX B cells. Molecular weight markers, lanes 1 and 11, (marked with lines) are, in descending order, 770, 220 and 72 bp. β -actin band appears as upper band (540 bp) and Fc γ RIIB1 RT-PCR product is the lower band (148 bp).

demonstrated that alloactivation of Fc γ RII/III⁺ CD4⁺ T cells induced *de novo* expression of Fc γ RIIB1 mRNA and cell surface protein, within 24 hours.

Induction of Fc γ RII/III expression on CD4⁺ T cells is blocked by anti-MHC class II monoclonal antibodies.

Previous observations have demonstrated that activation of some CD4⁺ T cell lines via MHC class II restricted antigen-presentation could induce FcR expression (15). Alloreactive CD4⁺ T cells are activated through direct engagement with the allo-MHC class II or via the presentation of processed alloantigens by syngeneic MHC class II (33,34). Because we used isolated CD4⁺ T cells, free of autologous APC, it was unlikely that the latter mechanism was active. However, mAb capable of blocking both H-2^d and H-2^k MHC class II were used to block Fc γ RIIB1 expression by B10.D2 CD4⁺ T cells. A 22 fold increase in the total number of Fc γ RIIB1⁺ CD4⁺ T cells was observed by 72 hours when purified CD4⁺ T cells were cocultured with the CH12.LX B cells alone (Table 4). However, when the anti-MHC class II mAb were present, only a 2 fold increase was observed (Table 4). Furthermore, the anti-MHC class II mAb blocked CD4⁺ T cell proliferation under the same conditions (data not shown). These data indicated that MHC class II recognition was required for the induction of Fc γ RII/III expression on CD4⁺ T cells.

Table 4. Induction of FcγRIIB1 expression on CD4⁺ T cells is blocked by anti-MHC Class II mAb.

anti-MHC Class II ^b	Added or recovered viable cells per culture ^a ($\times 10^{-3}$)		Mean Increase (\pm SD)
	Pre-culture	Post-culture	
-	78	1800	22(8)
-	6.6	130	
-	53	1700	
-	7.4	95	
+	78	120	2(0.6)
+	53	95	
+	7.4	15	
+	6.6	19	

- a) Data are the total number of viable CD4⁺ FcγRII/III⁺ T cells ($\times 10^{-3}$) added to or recovered per culture in 4 experiments. 10×10^6 total responder cells were added to each culture. 50×10^3 events were analyzed for each determination.
- b) Purified CD4⁺ T cells were cocultured with irradiated CH12.LX B for 72 hours in the presence or absence of 7.5 μ g/ml of each anti-MHC class II mAb (M5 and 10-2-16).

Activation of CD4⁺ T cells with anti-CD3 does not induce FcγRIIB1 expression.

The previous results indicated that activation of CD4⁺ T cells via MHC class II restricted alloantigen presentation induced *de novo* FcγRIIB1 expression. Therefore, we investigated whether direct TcR ligation could induce FcγRIIB1 expression. Initial experiments were conducted to determine the optimal conditions for activating CD4⁺ T cells with anti-CD3 and IL-2 (data not shown). Once activation conditions were established, CD4⁺ T cells were activated with anti-CD3 and IL-2 for 24 to 72 hours. No significant increases in the percent of FcγRII/III⁺ CD4⁺ T cells were observed at any of the time points after anti-CD3 activation, however, significant increases were observed after alloactivation with the CH12.LX B cells (Fig. 7). These results indicated that activation of CD4⁺ T cells with anti-CD3 could not induce FcγRII/III expression.

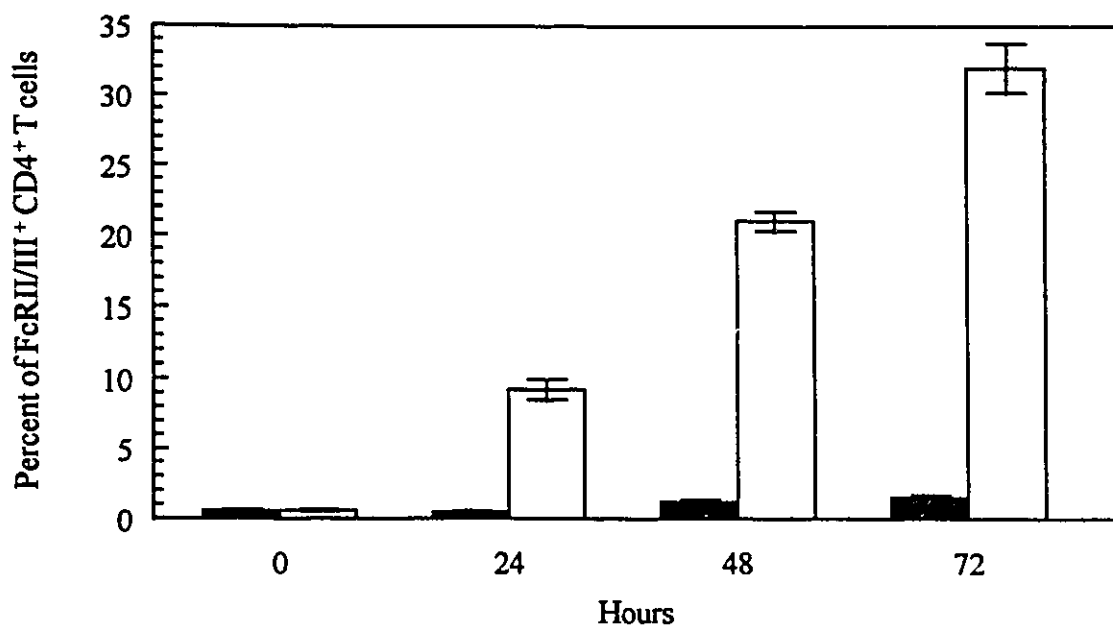


Figure 7 Activation of CD4⁺ T cells with anti-CD3 and IL-2 does not stimulate an increase in FcγRII/III⁺ CD4⁺ T cells.

CD4⁺ T cells were cultured with either irradiated CH12.LX B cells (white bar) or plate bound anti-CD3 plus rhIL-2 (20 U/ml) (black bar) for 24 or 72 hours. Data are percent values, \pm SD, from 4 experiments. 50×10^3 events were analyzed for each determination.

Discussion

It has been previously reported that CD8⁺ T cells and some CD4⁺ T cell lines can be activated to express FcR (3-5,7,15). In this study we showed definitively that normal splenic CD4⁺ T cells express FcγRIIB1. We investigated whether alloactivation of normal B10.D2 splenic CD4⁺ T cells could induce FcγRII/III expression. Following alloactivation significant increases in the number of FcγRII/III⁺ CD8⁺ T cells and FcγRII/III⁺ CD4⁺ T cells were detected. FcγRII/III⁺ CD4⁺ T cells were detected after 24 hours of alloactivation and the total number of these cells further increased after 48 and 72 hours. After 72 hours of activation, almost all (98±2%) CD8⁺ T cells were FcγRII/III⁺, whereas, only 16±3% of the CD4⁺ T cells were FcγRII/III⁺. The intensity of FcγRII/III staining on the CD8⁺ T cells and the CD4⁺ T cells was similar, indicating that both cells had similar amounts of surface FcγRII/III. The intensity of FcγRII/III staining on the T cells was also 10× less than that on B cells. In addition, the majority of the FcγRII/III⁺ CD4⁺ T cells failed to stain for either B220 or sIgM and therefore were not B cells. These results demonstrated that alloactivation of CD4⁺ and CD8⁺ T cells induced FcγRII/III expression. Furthermore, we observed that anti-MHC class II mAb could block CD4⁺ T cell proliferation and FcγRII/III expression, indicating that these cells were alloactivated and that the induction of FcγRII/III expression on CD4⁺ T cells was mediated via MHC class II restricted antigen presentation.

Since alloactivation resulted in increased numbers of FcγRII/III⁺ CD4⁺ T cells, we determined whether these cells had originated from the small number of initial splenic FcγRII/III⁺ CD4⁺ T cells, or if FcγRII/III expression had occurred *de novo*. Using FACS

sorting and RT-PCR, we showed that alloactivation of normal CD4⁺ T cells induced *de novo* FcγRIIB1 mRNA synthesis and surface expression within 24 hours. To accomplish this, we alloactivated FcγRII/III⁺ CD4⁺ T cells for 24 or 72 hours. FcγRII/III⁺ CD4⁺ T cells were detected after 24 hours and increased by 72 hours of culture. Furthermore, FcγRIIB1 mRNA was detected at both time points in the alloactivated CD4⁺ T cells, but not in the preculture FcγRII/III⁺ CD4⁺ T cells. Thus, alloactivation of CD4⁺ T cells induced *de novo* expression of FcγRIIB1 mRNA and surface expression, within 24 hours. These results argue against the possibility that the FcγRII/III detected on the CD4⁺ T cells was passively acquired from the CH12.LX B cells. This observation is also corroborated by the anti-MHC class II inhibition data, since the mAb block FcγRII/III expression even though the cells were still in coculture with the CH12.LX B cells.

The observed FcγRIIB1 expression on CD4⁺ T cells occurred by 24 hours of alloactivation, although no appreciable [³H]-thymidine uptake was apparent until after 48 hours. In addition, the number of cells expressing FcγRII/III increased by several fold within 24 hours, and thus could not be explained by normal lymphocyte proliferation. These results indicate that CD4⁺ T cell FcγRIIB1 expression was induced early after alloactivation and prior to DNA synthesis, and define FcγRIIB1 as an early activation marker of CD4⁺ T cells.

During this study we examined the normal B10.D2 spleen for FcγRII/III expression. Small numbers of CD4⁺ and CD8⁺ FcγRII/III⁺ T cells were detected in normal spleen and we observed that 6% of CD4⁺ T cells and ~2% of the CD8⁺ T cells were FcγRII/III⁺. Our results also indicated that there was a 7 fold higher number of FcγRII/III⁺ CD4⁺ T cells than

Fc γ RII/III⁺ CD8⁺ T cells in the B10.D2 spleen. However, the difference in numbers of these subsets could not be attributed to the absolute ratio of CD4⁺:CD8⁺ T cells in the normal spleen, which was 2:1. We examined T cell Fc γ RII/III expression using FACS analysis, as an extremely sensitive assay and analyzed 50×10^3 events for each sample. This ensured that significant numbers of Fc γ RII/III⁺ T cells were identified for an accurate calculation of the total number of Fc γ RII/III⁺ T cells. Both our data on alloactivated CD4⁺ T cells and other data with CD4⁺ T cell clones (15) suggest that only activated T cells express FcR. Therefore, these normal splenic Fc γ RII/III⁺ CD4⁺ T cells probably represent *in situ* activated CD4⁺ T cells.

It has been demonstrated that TcR activation of CD8⁺ T cells or some CD4⁺ T cell lines, via anti-CD3 or MHC class II restricted antigen presentation, can induce FcR expression (7,15). In this study we also show that activation of alloreactive CD4⁺ T cells via MHC class II induced Fc γ RIIB1 expression. However, activation of purified CD4⁺ T cells with anti-CD3 and IL-2 failed to induce significant numbers of Fc γ RII/III⁺ CD4⁺ T cells. Thus, TcR ligation alone was not sufficient to induce Fc γ RII/III expression on normal splenic CD4⁺ T cells. Other costimulatory signals provided from the CH12.LX B cells or allogeneic APC must be required for Fc γ RIIB1 expression by alloreactive CD4⁺ T cells.

During this study we noted 10 to 20 fold increases in the number of Fc γ RII/III⁺ CD4⁺ T cells after only 24 hours of alloactivation. However, others have calculated that only ~2% of all CD4⁺ and CD8⁺ T cells are alloreactive (35,36). These earlier studies define the frequency of alloreactive cell on the basis of limiting dilution of proliferating cells. Our results

indicate many more cells must be stimulated to produce Fc γ RIIB1, and this is blocked entirely by anti-MHC class II mAb. The high numbers of Fc γ RII/III⁺ CD4⁺ T cells we observed may be attributed to a higher frequency of CD4⁺ T cells that engage MHC class II to some degree resulting in the early induction of Fc γ RII/III expression. They do not preclude the fact that only a fraction of these cells would have a functional proliferative response.

We also observed that there was a marked enhancement in the induction of Fc γ RII/III expression on CD4⁺ T cells with increased purity of the CD4⁺ T cell preparation. When splenocytes were alloactivated for 72 hours, 16 \pm 3% were Fc γ RII/III⁺. Whereas, following activation of purified CD4⁺ T cells or FACS-sorted Fc γ RII/III⁻ CD4⁺ T cells, 26 \pm 5% and 33 \pm 2% were Fc γ RII/III⁺, respectively. These data suggest that within the normal spleen cultures, negative regulatory mechanisms may be present that are preventing the induction of Fc γ RIIB1 expression by the CD4⁺ T cells.

CH12.LX B cells provided the best stimulation of CD4⁺ T cells for the induction of Fc γ RIIB1 expression. This cell line is a proliferative B cell lymphoma (27,37) and it should express a variety of costimulatory molecules, such as B7 (38,39). Therefore, through direct contact with the CD4⁺ T cells, the CH12.LX could stimulate Fc γ RIIB1 expression using costimulation along with TcR engagement. The CH12.LX B cells could also influence Fc γ RIIB1 expression through the generation of various cytokines important to T cell activation, and in the generation of Th2 type CD4⁺ T cells (40,41). Finally, high concentrations of immunoglobulin have also been shown to induce FcR expression, including

Fc γ RII/III, on normal CD8⁺ T cells *in vitro* (2,10). Therefore, the expression of Fc γ RIIB1 on CD4⁺ T cells could be augmented by the presence of IgG2b produced by this cell line of CH12.LX (37). IgG2b binds with the highest affinity for Fc γ RIIB1 of all the IgG subclasses (1). Currently, we are conducting studies to evaluate the role of the B cell, costimulatory molecules, cytokines and soluble immunoglobulin in the expression of Fc γ RII/III by CD4⁺ T cells.

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Chapter Five

FcγRIIB1 expression on CD4⁺ T cells

The following article entitled "CH12.LX B cells secrete soluble factors that enhance FcγRII/III expression on alloactivated CD4⁺ T cells" is a manuscript in preparation. This study examined some of the factors that may be involved in regulating the induction of FcγRIIB1 expression on alloactivated CD4⁺ T cells.

This work presented in this study was performed by the author of the thesis. This paper was written by the author of this thesis. Drs. Snider and Ernst supervised this study.

TITLE:

CH12.LX B cells secrete soluble factors that enhance Fc γ RII/III expression on alloactivated CD4⁺ T cells.

RUNNING TITLE:

B cells secrete factors that enhance CD4⁺ T cell Fc γ RII/III expression.

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Abstract

We present evidence that CH12.LX B cells generate soluble factors that promote the induction of Fc γ RII/III expression on the surface of alloactivated normal peripheral CD4⁺ T cells. Splenic CD4⁺ T cells from B10.D2 mice, when alloactivated with IgG2b⁺, IgG3⁺ or IgA⁺ CH12.LX B cells, expressed surface Fc γ RII/III, as defined by mAb 2.4G2. Alloactivation of purified CD4⁺ T cells with IgG2b⁺ B cells resulted in a 25 to 60 fold increase in the total number of Fc γ RII/III⁺ CD4⁺ T cells, while IgG3⁺ and IgA⁺ B cells induced a 7 fold and 4 fold increase, respectively. The IgG2b⁺ CH12.LX B cell line secreted a soluble factor that could enhance the induction of Fc γ RII/III⁺ CD4⁺ T cells, however, a purified IgG2b myeloma protein could not enhance the induction of Fc γ RII/III expression on alloactivated CD4⁺ T cells. Finally, activation of CD4⁺ T cells with IL-1 did not induce Fc γ RII/III expression. These studies demonstrate that CH12.LX B cells secrete soluble factors that promote the induction of Fc γ RII/III expression on alloactivated CD4⁺ T cells and Fc γ RII/III expression may not be dependent upon the Ig isotype of the CH12.LX B cell.

Introduction

Expression of Fc receptors (FcR), for each of the five immunoglobulin (Ig) isotypes, have been demonstrated on both human and murine T cells (1). Both B cells and Ig have been shown to induce FcR expression on CD8⁺ T cells. Initial studies that investigated murine T cell FcR expression, demonstrated that FcR⁺ CD8⁺ T cells could be generated *in vivo* through the adoptive transfer of either B cell plasmacytomas or hybridomas (2-5) or by elevating serum Ig levels (2,4,6). We have also observed that FcR⁺ CD8⁺ T cells can be generated *in vitro* by coculturing splenic lymphocytes with allogeneic splenocytes or a B cell lymphoma (7). FcR⁺ CD8⁺ T cells have also been observed in patients that have elevated levels of serum Ig (8,9) and these cells can also be generated *in vitro* by coculturing human PBL with allogeneic B cell lines (10). Furthermore, it has been observed in mouse and human that the isotype-specificity of the FcR expressed on the CD8⁺ T cells corresponded with the isotype of the Ig generated by the B cells (2-6,8,9).

Little is known about the role of B cells and Ig in the induction of FcR expression on CD4⁺ T cells. Elevated numbers of Fc α R⁺ CD4⁺ T cells have been shown to be present in murine Peyer's patches (11) and recently we have observed that coculturing IgG2b⁺ CH12.LX B cells with purified CD4⁺ T cells could induce *de novo* Fc γ RIIB1 mRNA synthesis and surface expression (7). In that study we observed that the induction of Fc γ RIIB1 expression, by the CH12.LX B cell lymphoma, was MHC class II restricted, however, direct TcR ligation with anti-CD3 was not sufficient to induce Fc γ RIIB1 expression (7).

Therefore, we set out to better define the role of the B cell and Ig isotype in the induction of Fc γ RII/III expression on mature peripheral CD4⁺ T cells using *in vitro* alloactivation. Based on FACS analysis, we demonstrate that activation of normal splenic CD4⁺ T cells with allogeneic B cells induces Fc γ RII/III expression. However, the induction of Fc γ RII/III expression is not strictly dependent upon the Ig isotype of the CH12.LX B cell. In addition we show that CH12.LX B cells generate soluble factors that promote the induction of Fc γ RII/III expression on normal CD4⁺ T cells, however, soluble IgG2b did not induce Fc γ RII/III expression.

Materials and Methods

Mice.

Female B10.A and B10.D2N mice (Jackson Laboratories, Bar Harbour, MA) were used between 8-10 weeks of age and housed under conventional conditions at McMaster University.

Antibodies and Cell Lines

The monoclonal antibodies purchased for FACS analysis were 2.4G2 (12) anti-Fc γ RII/III-PE, (Pharmagen, San Diego, CA), goat-anti-mouse IgM-PE (Jackson ImmunoResearch, West Grove, PA), and goat-anti-mouse IgM-biotin, (Southern Biotechnology, Birmingham, AL). Others were isolated on either protein-G or protein-A columns from tissue-culture supernatants or ascites and labelled with FITC or biotin in our lab using standard techniques (13). These included 6B2 (14), anti-B220; 145-2C11 (15), anti-CD3 (CRL 1975, ATCC, Rockville, MD); GK1.5 (16), anti-CD4 (TIB 207, ATCC); and 53-6.72 (17), anti-CD8 (TIB 105, ATCC). The mAb YTS 169.4, anti-CD8 α (Cedarlane, Hornsby, Ontario), J11d.2 (18), anti-heat stable antigen (HSA), (TIB 183, ATCC) and M5/114.15.2 (19), anti-I-A^{b,4q} and anti-I-E^{4k}, (TIB 120, ATCC) were used to isolate CD4⁺ T cells. The IgG2b myeloma protein (Sigma, St. Louis, MO) was purified using on a Altex sepharogelTM-TSK column (4000 SW) (Beckman, Mississauga, ON) on a Gilson HPLC (Madel Scientific, Lachine, PQ). The CH12.LX B cell lines that secrete IgG2b, IgG1, IgG3

or IgA were generously provided by Dr. Geoffrey Houghton, UNC (20,21). All cells were cultured in RPMI 1640 supplemented with penicillin, streptomycin, L-glutamine, sodium pyruvate, HEPES, 2-ME and 10% FCS (10%FCS-RPMI).

Detection of IgG2b

Soluble IgG2b generated by irradiated IgG2b⁺ CH12.LX B cell line was detected and quantified using a standard sandwich ELISA. Assays were performed in duplicate in 96 well Nunc Maxisorp[®] microtitre plates. Wells were coated with 50 µl of goat anti-mouse IgG (2 µg/ml) (Southern Biotechnology) diluted in BBS (0.2M boric acid, 0.05M borax: pH 8.64), for 3 hours at 22° C. Plates were blocked for 1 hour at 22° C with 150 µl of TBS-BSA (10 mg/ml) (0.02M Tris, 0.15M NaCl₂, 0.2% NaN₃: pH8.0). 50 µl of purified IgG2b myeloma protein (see above) and diluted culture supernatants were added to the wells and incubated for 18 hours at 4° C. The ELISA plates were then washed 3 times with 170 µl of TBS-Tween (0.5%). 50 µl of biotinylated goat anti-mouse IgG2b (1µg/ml) (Southern Biotechnology) was added to each well and incubated for 2 hours at 22° C. The ELISA plates were washed again and then 50 µl of p-NNP (Sigma) diluted in ethanolamine buffer was added to each well. The ELISA plates were incubated for 15 to 30 minutes at 37° C, and subsequently read in an Titertek Multiskan[®] Plus Mk II ELISA plate reader (ICN-Flow, Mississauga, ON) at 405 nm. The mean OD of the purified IgG2b were used to generate a standard curve from which the concentrations of IgG2b in the CH12.LX culture supernatants were interpolated.

Isolation of CD4⁺ T cells

Splenic CD4⁺ T cells were isolated by incubating nylon wool nonadherent B10.D2 spleen cells at 10×10^6 per ml of HBSS containing 5 μ g of anti-I-A^d, a 1:5 dilution of anti-HSA supernatant and a 1:300 dilution of anti-CD8 (YTS 169.4), for 45 min at 4° C. The cells were subsequently incubated with a 1:10 dilution of rabbit complement (Cedarlane) for 1 hour at 37° C. The splenic CD4⁺ T cells were then analyzed for viability and B220, IgM, CD3, CD4, CD8 and Fc γ RII/III surface expression by FACS analysis. The purified CD4⁺ T cells were 80 \pm 5% CD4⁺, 79 \pm 4% CD3⁺, 3 \pm 3% IgM⁺, 3 \pm 2% B220⁺, 0.2 \pm 0.2% CD8⁺ and 96 \pm 3% viable prior to coculture (Fig. 1). After allogeneic coculture with CH12.LX B cells for 72 hours the cells were 96 \pm 4% CD4⁺, 96 \pm 2% CD3⁺, 1 \pm 1% IgM⁺, 1 \pm 1% B220⁺, and 94 \pm 5% viable (data from 10 experiments) (Fig. 1).

Activation of CD4⁺ T cells to Induce Fc γ RII/III Surface Expression

Purified B10.D2 CD4⁺ T cells (10×10^6) were cultured with 5×10^6 irradiated CH12.LX B cells (5000 rads) for 24 to 72 hours, in 6 well plates (Corning Inc., Corning, NY) containing 10%FCS-RPMI. The ratio of responder CD4⁺ T cells to stimulators was determined for optimal CD4⁺ T cell proliferation (see below). In some experiments 2×10^6 purified CD4⁺ T cells were activated, for 24 to 72 hours, with rIL-1 (200 U/ml) (Boehringer Mannheim, Laval, Quebec) in 6 well plates (Corning).

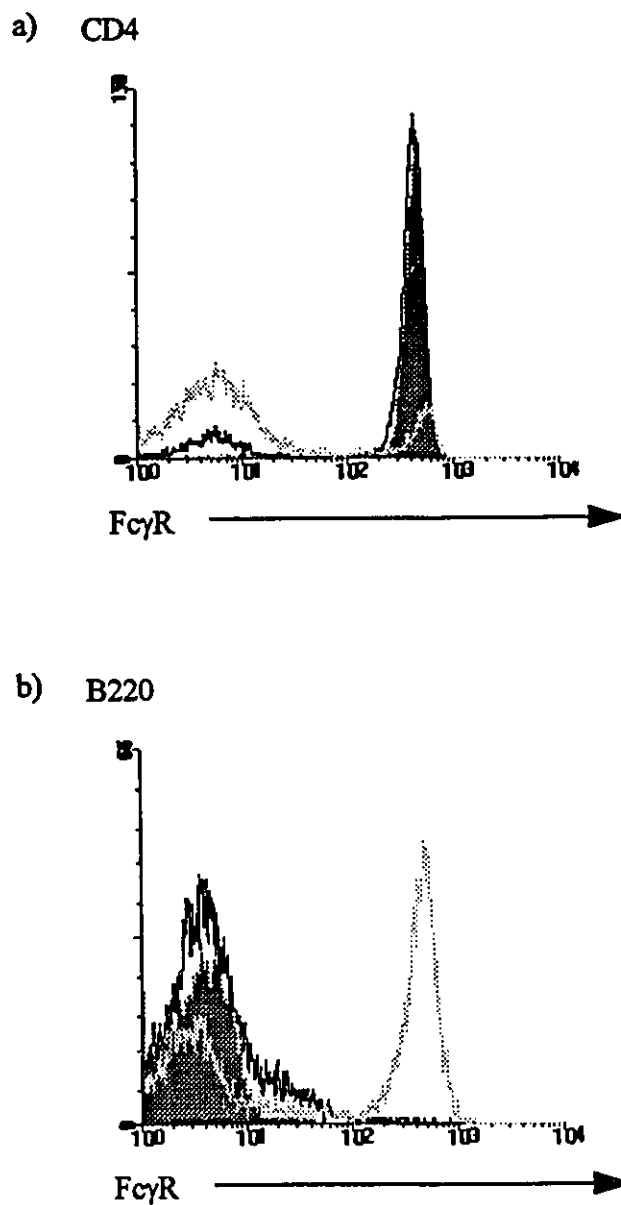


Figure 1 Composition of the CD4⁺ T cell preparations were examined before and after alloactivation with CH12.LX B cells.

The data are from one representative experiment showing CD4 staining (a), B220 staining (b) on unseparated spleen (dotted line) or purified CD4⁺ T cells before alloactivation (solid line) and after 72 hours of alloactivation (solid grey). Splenic lymphocytes and CD4⁺ T cells were 95±5% viable (n=10) by PI staining.

Proliferation Cultures and Assay

CD4⁺ T cell proliferative responses were determined by [³H]-thymidine incorporation (1.0 µCi/well) (NEN, Boston, MA). Various numbers (8×10^5 to 6×10^3) of CD4⁺ T cells were incubated with (8×10^5 to 1×10^5) CH12.LX B cells irradiated with 5000 rads. These cultures were conducted in 96 well flat bottom plates (Corning) in 10%FCS-RPMI. Cultures were harvested using a PHDTM cell harvester (Cambridge Technologies, Inc., Cambridge, MA). Samples were counted on a Beckman LS-5801 beta counter (Beckman Instruments, Mississauga, Ontario).

Flow Cytometry Analysis of CD4⁺ T cells

CD4⁺ T cells were suspended in 50 µl of PBS/0.2% BSA/0.1% NaN₃ (PBS/BSA) at a concentration of 1×10^7 cells/ml, with 75 µl of mixtures of up to 3 labelled diluted mAb (optimal titrations), at 4° C for 30 minutes. The cells were subsequently washed and then incubated in 50 µl PBS/BSA with 10 µl of strepavidin-PerCP (Becton Dickinson, San Jose, CA) at 4° C for 15 minutes, in order to detect the biotinylated mAb. Cells were washed again, suspended in 500 µl of PBS/BSA and analyzed on a FACScan (Becton Dickinson). For analysis of FcγRII/III expression on CD4⁺ T cells, 5×10^4 events were collected. To identify FcγRII/III⁺ CD4⁺ T cells, cells were stained with anti-CD4-FITC, anti-FcγRII/III-PE and anti-B220-biotin or anti-IgM-biotin. CD4⁺ T cell viability was determined using propidium iodide staining with anti-CD4-FITC. Compensation adjustments were done on sets of single-stained cells. Flow cytometry data was analyzed utilizing PC-LYSYS software (Becton

Dickinson).

Calculation of Total Number of CD4⁺ FcγRII/III⁺ T cells

Flow cytometry data, obtained from analysis of triple staining with anti-CD4-FITC, anti-FcγRII/III-PE and anti-B220- or anti-IgM-biotin-streptavidin-PerCP, was used to quantitate FcγRII/III⁺ CD4⁺ T cells. Lymphocytes were gated by forward and side scatter parameters. A second gate defined all positive CD4-FITC staining cells. Combining the two gates allowed analysis of CD4⁺ T cells for expression of FcγRII/III and B220 (or IgM) in the second and third colours. The total number of CD4⁺ FcγRII/III⁺ lymphocytes in the analyzed sample and the small number of contaminating B cells (defined as B220⁺ or IgM⁺ and FcγRII/III⁺) were determined by two colour analysis. The number of contaminating B cells was subtracted from the number of FcγRII/III⁺ CD4⁺ lymphocytes yielding the actual number of true FcγRII/III⁺ CD4⁺ T cells within the analyzed sample. The percent of FcγRII/III⁺ CD4⁺ T cells in the analyzed sample was determined by dividing the total number of FcγRII/III⁺ CD4⁺ T cells by the total number of lymphocytes in the scatter gate. A total viable lymphocyte count was made based on trypan blue exclusion and counting on a haemocytometer. The total number of FcγRII/III⁺ CD4⁺ T cells was then calculated by multiplying the total number of viable lymphocytes harvested from the coculture by the percent of FcγRII/III⁺ CD4⁺ T cells.

Results

Alloactivation of CD4⁺ T cells with IgG2b⁺, IgG3⁺ or IgA⁺ CH12.LX B cells induces FcγRII/III⁺ expression.

We have shown previously that activation of CD4⁺ T cells with allogeneic CH12.LX B cells could induce *de novo* FcγRIIB1 expression (7). The subline of the B cell used to stimulate the CD4⁺ T cells expressed IgG2b Ig. This IgG subclass is known to bind to FcγRII/III with higher affinity than other IgG subclasses or Ig isotypes (e.g. IgA) (1). Therefore, we investigated whether the induction of FcγRII/III expression on CD4⁺ T cells, by CH12.LX B cells, was dependent upon the Ig isotype of the stimulating B cell. Initial experiments were conducted to determine the conditions required for the alloactivation of the B10.D2 CD4⁺ T cells with the irradiated CH12.LX B cells. CD4⁺ T cells from B10.D2 mice were chosen as the responder population since B10.D2 mice are allogeneic (at both MHC class I and II loci) to B10.A mice from which the CH12.LX B cell lines were derived (20). Data from these experiments demonstrated that the irradiated IgG2b⁺, IgG3⁺ and IgA⁺ CH12.LX B cells could stimulate B10.D2 CD4⁺ T cells to proliferate (Fig. 2). However, the subclones could not equally allostimulate the CD4⁺ T cells, on a per cell basis. In fact, the IgG1⁺ subline did not stimulate any CD4⁺ T cell proliferation (Fig. 2).

Once MLR conditions were established, purified CD4⁺ T cells were co-cultured with optimal numbers of IgG2b⁺, IgG3⁺, or IgA⁺ CH12.LX B cells for 72 hours, harvested and analyzed for FcγRII/III expression using the mAb 2.4G2 (12). A 42.0±13 fold (n=5) increase in the total number of FcγRII/III⁺ CD4⁺ T cells was observed when the CD4⁺ T cells were

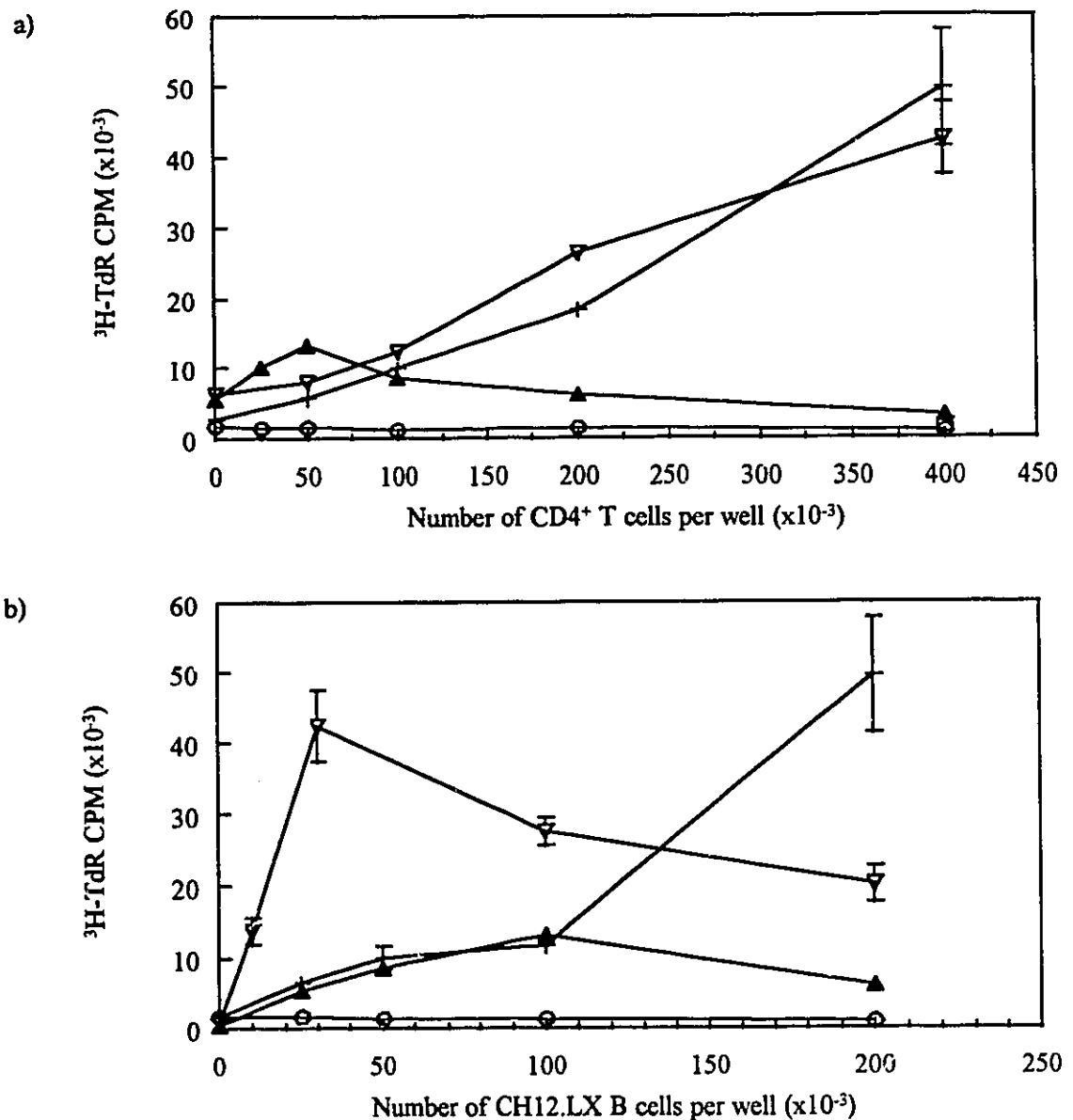


Figure 2 CH12.LX B cell subclones were evaluated for their ability to stimulate B10.D2 CD4^+ T cell proliferation.

In figure A, various numbers (x-axis) of B10.D2 CD4^+ T cells were cultured alone (□) or with 200×10^3 IgG2b^+ (+), 100×10^3 IgG1^+ (O), 100×10^3 IgG3^+ (▲) or 30×10^3 IgA^+ (▽) CH12.LX B cells for 96 hours. In figure B, various numbers (x-axis) of IgG2b^+ (+), IgG1^+ (O), or IgA^+ (▽) CH12.LX B cells were cultured with 400×10^3 B10.D2 CD4^+ T cells for 96 hours. IgG3^+ (▲) CH12.LX B cells were cultured with 50×10^3 B10.D2 CD4^+ T cells. Cultures were pulsed with [^3H]-thymidine 24 hours prior to harvesting. Data are mean CPM values, \pm SD, from one representative experiment.

cocultured with the IgG2b⁺ subline (Fig 3b,c, Table 1). When CD4⁺ T cells were cocultured with the IgG3⁺ subline, a 7.3 ± 0.5 fold increase (n=9) in the total number of FcγRII/III⁺ CD4⁺ T cells was detected and a 4.3 ± 0.8 fold increase (n=5) was detected when the CD4⁺ T cells were cocultured with IgA⁺ subline, (Fig. 3d,e,f,g, Table 1). These results indicated that FcγRII/III expression could be induced on CD4⁺ T cells regardless of the isotype or IgG subclass expressed by the allogeneic CH12.LX B cells, although the highest induction occurred using the IgG2b⁺ subline.

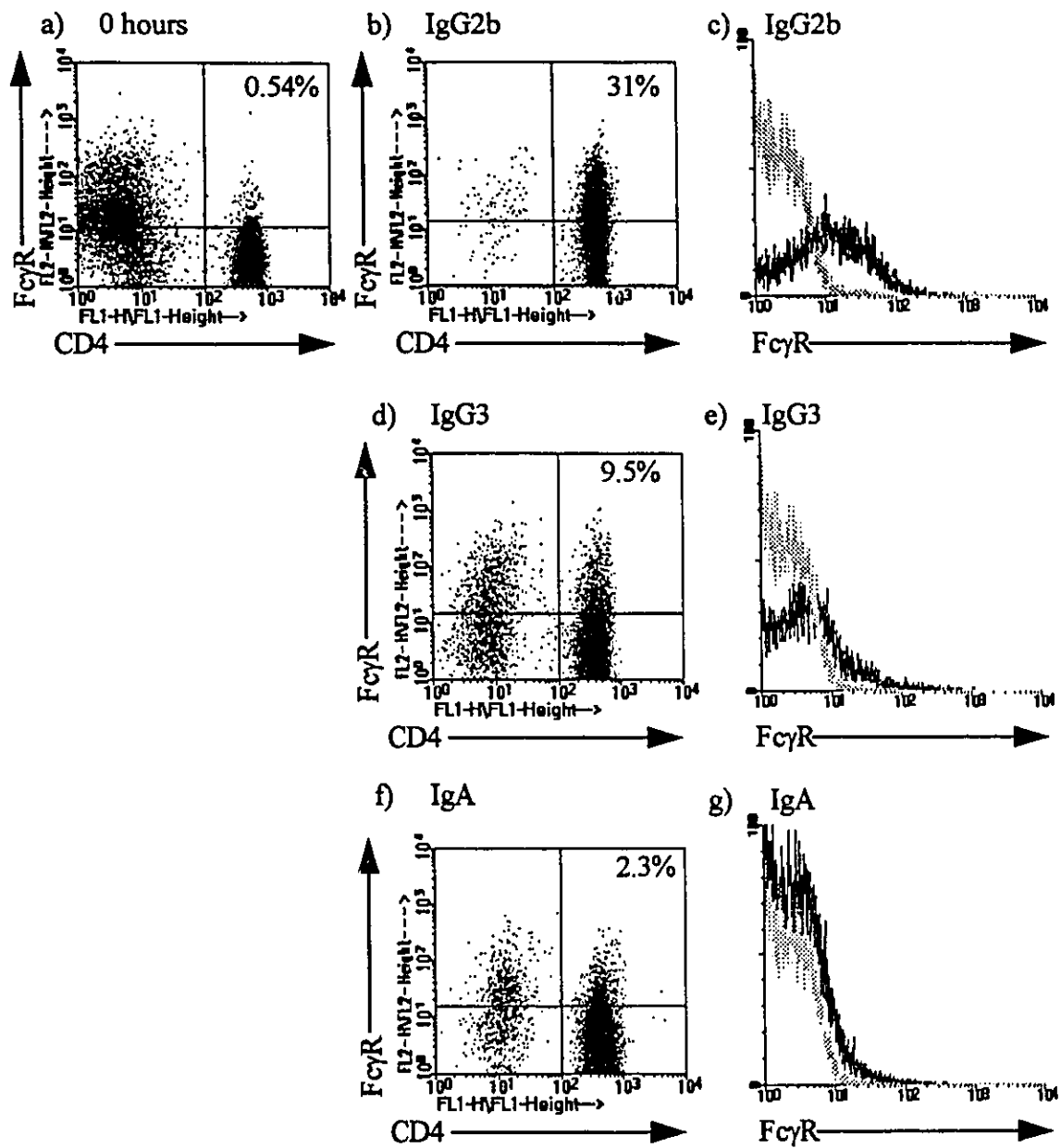
IgG2b⁺ CH12.LX B cells generate soluble factors that promote the induction of FcγRII/III expression on alloactivated CD4⁺ T cells.

The preceding experiment showed that the induction of FcγRII/III expression on CD4⁺ T cells was not dependent upon the Ig isotype of the CH12.LX B cells. However, the greatest increase in the number of FcγRII/III⁺ CD4⁺ T cells was observed after using the IgG2b⁺ subline. Therefore, we investigated whether the IgG2b⁺ CH12.LX B cell line was generating soluble factors that promoted the induction of FcγRII/III expression on CD4⁺ T cells. Purified CD4⁺ T cells were cocultured with IgA⁺ CH12.LX B cells in transwells for 72 hours, with or without the IgG2b⁺ subline present in the upper transwell. The use of transwells ensured that the alloactivated CD4⁺ T cells would be exposed to any soluble factors generated by the irradiated IgG2b⁺ subline but not in direct contact with them. The CD4⁺ T cells were cultured with IgA⁺ CH12.LX B cells at two different effector:target ratios, 27:1 and 2:1. The ratio of 27:1 was used to stimulate a proliferative response comparable to the

Table 1. Alloactivation of CD4⁺ T cells with CH12.LX B cells induces FcγRII/III expression.

Isotype of B cell ^b	Added or recovered viable cells per culture* (×10 ⁻³)		Mean Increase (±SD)
	Pre-culture	Post-culture	
IgG2b	1.0	56.9	47(13)
	1.0	63.8	
	25	1183	
	3.7	125	
	6.0	202	
IgG1	94	148	1.4(0.6)
	88	206	
	59	59	
	68	68	
	50	67	
IgG3	10	73	7.4(0.5)
	64	439	
	47	366	
	48	378	
	65	453	
IgA	7.4	36	4.6(0.8)
	29	131	
	51	221	
	38	222	
	44	159	

- a) Data are the total number of viable CD4⁺ FcγRII/III⁺ T cells (×10⁻³) added to or recovered per culture in 10 experiments. 10×10⁶ total responder cells were added to each culture. 50×10³ events were analyzed for each determination.
- b) Purified CD4⁺ T cells were cocultured with irradiated CH12.LX B cells.



Legend to Figure 3

Alloactivation of purified CD4⁺ T cells with CH12.LX B cells induces FcγRII/III expression. The data are from one representative experiment. The dotplots show FcγRII/III-PE (y-axis) and CD4-FITC (x-axis) prior to coculture (a) and after 72 hours of coculture with IgG2b⁺ (b), IgG3⁺ (d) or IgA⁺ (f) CH12.LX B cells. The histograms show FcγRII/III-PE staining on CD4⁺ T cells prior to (dotted line) or after 72 hours of coculture (solid line) with IgG2b⁺ (c), IgG3⁺ (e) or IgA⁺ (g) CH12.LX B cells. CD4⁺ T cells were 95±3% viable by PI staining.

proliferative response induced by the IgG2b⁺ subline at its optimal ratio of 2:1 (Fig. 2a,b). The effector:target ratio of 2:1 assured that the CD4⁺ T cells received cellular contact with the IgA⁺ subline equivalent to that received during a typical culture with the IgG2b⁺ subline. An 18.8 ± 4.5 (n=5) fold increase in the number of FcγRII/III⁺ CD4⁺ T cells was observed when CD4⁺ T cells were cocultured with IgG2b⁺ CH12.LX B cells for 72 hours (Table 2). A 5.1 ± 0.4 (n=5) fold increase was detected when CD4⁺ T cells were cocultured with IgA⁺ B cells at the ratio of 27:1 and a 17 ± 1.3 (n=4) fold increase was observed when the CD4⁺ T cells were cocultured with the IgA⁺ CH12.LX B cells at the ratio of 2:1 (Table 2). However, when the IgG2b⁺ subline was present in the upper transwell, a 4.2 ± 0.2 (n=5) fold increase was observed at the ratio of 27:1 and a 17 ± 3.7 (n=5) fold increase was observed at the ratio of 2:1 (Table 2). These results indicated that the irradiated IgG2b⁺ CH12.LX B cells were generating factors that enhanced the induction of FcγRII/III expression on alloactivated CD4⁺ T cells.

Soluble IgG2b does not induce FcγRII/III expression on alloactivated CD4⁺ T cells.

Soluble Ig has been shown to induce FcR expression on CD8⁺ T cells (6,9,10,22-24) and we tested and found soluble IgG2b (0.5 μg/ml) in CH12.LX supernatants (data not shown). Therefore, we investigated whether IgG2b was augmenting FcγRII/III expression. CD4⁺ T cells were cocultured with the IgA⁺ subline at the ratio of 2:1, with or without purified IgG2b for 72 hours. The amount of purified soluble IgG2b added to the coculture was equal to 1 to 10 times the amount of soluble IgG2b generated by 5×10^6 irradiated IgG2b⁺

Table 2. IgG2b⁺ CH12.LX B cells secrete factors that promote the induction of Fcγ RII/III expression on alloactivated CD4⁺ T cells.

CH12.LX Ig Isotype ^b	Transwell culture	Added or recovered viable cells per culture ^a (×10 ³)		Mean Increase (±SD)
		Pre-culture	Post-culture	
IgG2b	-	4.4	93	18(3.7)
		6.9	141	
		7.9	133	
		5.5	70	
IgA (27:1)	-	4.4	25	5.1(0.4)
		5.5	26	
		6.9	33	
		7.9	40	
IgA (27:1)	IgG2b	4.4	78	17(1.3)
		5.5	101	
		7.9	121	
		6.9	119	
IgA (2:1)	-	4.4	19	4.4(0.2)
		5.5	23	
		7.9	36	
IgA (2:1)	IgG2b	4.4	70	17(3.7)
		5.5	111	
		7.9	101	
		6.9	141	

- a) Data are the total number of viable CD4⁺ Fcγ RII/III⁺ T cells (×10³) added to or recovered per culture in 4 experiments. 10×10⁶ total responder cells were added to each culture. 50×10³ events were analyzed for each determination.
- b) Purified CD4 T cells were cocultured with irradiated IgA⁺ CH12.LX B cells, at ratios of 27:1 or 2:1, for 72 hours in the presence or absence of IgG2b⁺ CH12.LX B cells.

CH12.LX B cells within 72 hours (data not shown). A 24.1 ± 5 fold ($n=4$) increase in the total number of Fc γ RII/III⁺ CD4⁺ T cells was detected after the CD4⁺ T cells were cocultured with the IgG2b⁺ subline (Fig. 4). A 3.14 ± 2.5 fold ($n=4$) increase was detected when CD4⁺ T cells were cultured with the IgA⁺ subline alone (Fig. 4). Comparable increases in the total number of Fc γ RII/III⁺ CD4⁺ T cells were also observed when CD4⁺ T cells were cocultured with the IgA⁺ subline and 0.5 to 5.0 μ g/ml of purified IgG2b (Fig. 4). These results indicated that soluble IgG2b could not augment Fc γ RII/III expression on CD4⁺ T cells that were activated by IgA expressing B cells.

Activation of CD4⁺ T cells with IL-1 does not induce Fc γ RII/III expression.

It has been reported that only CD4⁺ T cells that generate Th2 cytokines can express FcR (25) and it has also been shown that Th2 CD4⁺ T cells express IL-1R (26-28). Furthermore, it has been demonstrated that activation of CD4⁺ T cells with IL-1 could induce the generation of IgGBF (29), which we have shown to be soluble Fc γ RII/III (30). Therefore, we investigated whether the activation of CD4⁺ T cells with IL-1 could induce Fc γ RII/III expression. Purified CD4⁺ T cells were incubated with 200 U/ml of rIL-1 for 24 or 72 hours. This concentration of rIL-1 has been shown to activate CD4⁺ T cells to generate IgGBF *in vitro* (29). No significant increases in the number of Fc γ RII/III⁺ CD4⁺ T cells were observed at either of the time periods (Table 3), however, soluble Fc γ RII/III was detected at both time points (data not shown). These results indicated that activation of CD4⁺ T cells with IL-1 could not induce Fc γ RII/III surface expression.

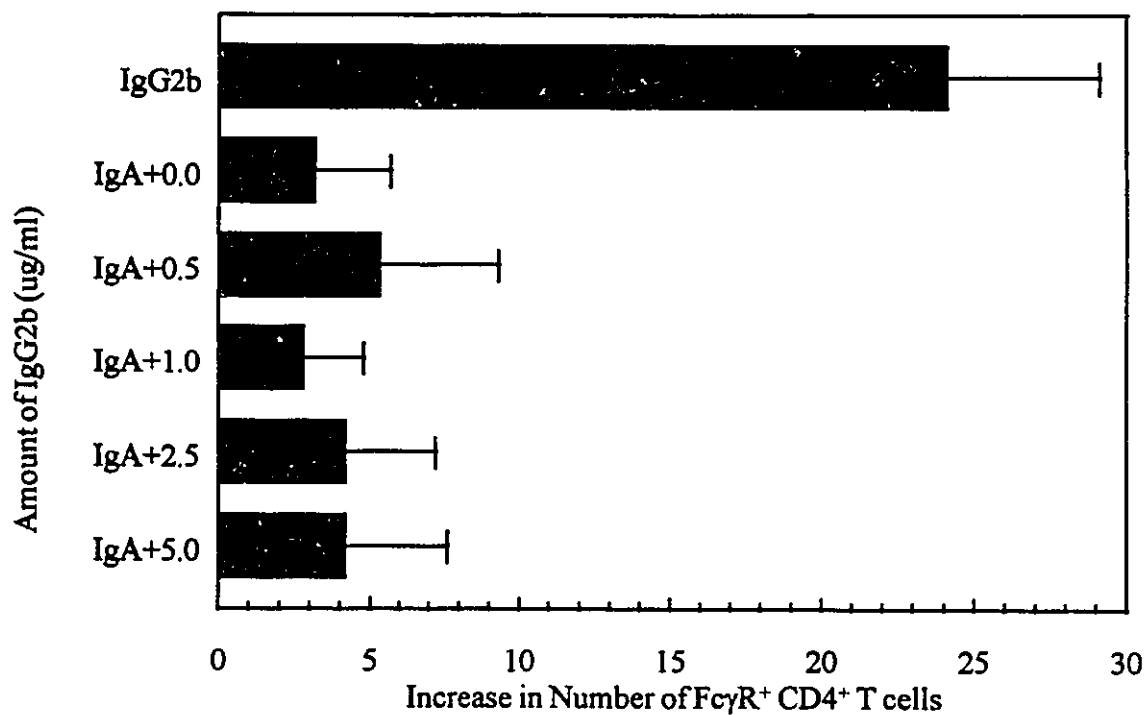


Figure 4 Soluble IgG2b does not induce FcγRII/III expression on alloactivated CD4⁺ T cells.

Purified CD4⁺ T cells were cocultured with IgA⁺ CH12.LX B cells in the presence (0.5 to 5.0 μg/ml) or the absence of purified soluble IgG2b for 72 hours. Control was IgG2b CH12.LX. Data are mean fold increases in the number of FcγRII/III⁺ CD4⁺ T cells, ±SD, from 2 experiments.

Table 3. Activation of CD4⁺ T cells with rIL-1 does not induce FcγRII/III expression.

Time in culture ^b	Added or recovered viable cells per culture ^a (×10 ³)		Mean Increase (±SD)
	Pre-culture	Post-culture	
24	1.0	0.8	1.0(0.3)
	1.0	0.6	
	47	58	
	51	64	
72	1.0	1.3	1.2(0.4)
	1.0	0.7	
	47	62	
	51	80	

- a) Data are the total number of viable CD4⁺ FcγRII/III⁺ T cells (×10³) added or recovered per culture in 4 experiments. 10×10⁶ total responder cells were added to each culture. 50×10³ events were analyzed for each determination.
- b) Purified CD4⁺ T cells were activated with 200 U/ml of rIL-1 for 24 or 72 hours.

Discussion

In this study we demonstrate that alloactivation, with CH12.LX B cells, induces FcγRII/III expression on CD4⁺ T cells. Furthermore, we show that the CH12.LX B cells generate soluble factors which augment FcγRII/III expression on alloactivated CD4⁺ T cells.

It has been previously reported that activation of CD8⁺ T cells, *in vivo* and *in vitro*, with B cell lines or Ig, induces FcR expression and the isotype-specificity of the FcR corresponded with the isotype of the Ig (2-7,10). Therefore, we investigated whether the induction of FcγRII/III expression on CD4⁺ T cells by CH12.LX B cells was isotype-specific. Following alloactivation with isotype-switch variants of the CH12.LX B cell line, significant increases in the number of FcγRII/III⁺ CD4⁺ T cells were detected. After 72 hours of alloactivation with the IgG2b⁺ subline a 42.0±13 fold increase in the total number of FcγRII/III⁺ CD4⁺ T cells was observed. Furthermore, a 7.3±0.5 fold increase in the total number of FcγRII/III⁺ CD4⁺ T cells was observed following alloactivation with the IgG3⁺ subline and a 4.3±0.8 fold increase was detected after alloactivation with the IgA⁺ subline. These results demonstrated that alloactivation of CD4⁺ T cells, with CH12.LX B cells, induced FcγRII/III expression and suggested that the induction of FcγRII/III expression may be isotype-nonspecific.

Since the greatest increase in the number of FcγRII/III⁺ CD4⁺ T cells was observed following alloactivation with IgG2b⁺ CH12.LX B cells, we investigated whether the IgG2b⁺ subline was generating soluble factors that augmented FcγRII/III expression on alloactivated CD4⁺ T cells. CD4⁺ T cell were cultured with IgA⁺ CH12.LX B cells at two different

effector:target ratios, 27:1 and 2:1, with the IgG2b⁺ subline present in an upper transwell. The ratio of 27:1 stimulated a proliferative response comparable to the proliferative response induced by the IgG2b⁺ subline at its optimal ratio of 2:1. The ratio of 2:1 assured that the CD4⁺ T cells received cellular contact equivalent to that received during coculture with the IgG2b⁺ subline. FcγRII/III⁺ CD4⁺ T cells were detected following alloactivation with the IgA⁺ subline, however, when the IgG2b⁺ subline was present, we observed a further 3 to 4 fold increase in the total number of FcγRII/III⁺ CD4⁺ T cells after alloactivation. These results indicated that the irradiated IgG2b⁺ CH12.LX B cells were generating soluble factors that enhanced FcγRII/III expression on alloactivated CD4⁺ T cells.

It has been demonstrated, *in vivo* and *in vitro*, that elevated levels of soluble Ig can induce FcR expression on CD8⁺ T cells (2,4,6,10,22,24) and soluble IgG2b was detected in the supernatants of the irradiated the IgG2b⁺ subline. Therefore, we investigated whether soluble IgG2b could enhance FcγRII/III expression on alloactivated CD4⁺ T cells. CD4⁺ T cells were alloactivated with the IgA⁺ subline in the presence of 0.5 to 5 μg/ml of purified soluble IgG2b for 72 hours. This concentration of IgG2b was equivalent to 1 to 10 times the amount of IgG2b generated by irradiated IgG2b⁺ CH12.LX within 72 hours. No significant increase in the number of FcγRII/III⁺ CD4⁺ T cells was observed when IgG2b was added to the cultures. This result indicated that soluble IgG2b could not enhance FcγRII/III expression on CD4⁺ T cells alloactivated with IgA⁺ CH12.LX B cells. Although the concentrations of Ig used in this study were physiologically relevant, it is possible that insufficient IgG2b was added to the cultures, since previous reports had demonstrated that

concentrations of 300 to 500 $\mu\text{g/ml}$ of Ig were required to induce FcR expression on splenic CD8^+ T cells (10,22-24). It has also been reported that elevated levels of IgA (300 $\mu\text{g/ml}$) can down regulate Fc γ RII/III expression on CD8^+ T cells (31). Therefore, the inability of IgG2b to enhance Fc γ RII/III expression on the alloactivated CD4^+ T cells may be due to endogenous IgA present during the experiment. The role of Ig and Ig isotype in regulating Fc γ RII/III expression on CD4^+ T cells remains undefined and clearly requires further investigation.

Since, it has been previously observed that activation of CD4^+ T cells with rIL-1 induces the generation of sFc γ RII/III (29), we investigated whether rIL-1 could induce Fc γ RII/III surface expression on CD4^+ T cells. No increase in the number of Fc γ RII/III $^+$ CD4^+ T cells was observed after 24 or 72 hours of activation with IL-1, however, sFc γ RII/III was detected (data not shown). These results indicated that IL-1 was not involved in the induction of Fc γ RII/III surface expression. However, our data indicated that the CH12.LX IgG2b $^+$ B cell line was secreting soluble factor(s) that influenced Fc γ RII/III expression on the alloactivated CD4^+ T cells. CH12.LX B cells reportedly secrete IL-3,4,6 and 10, $\text{TNF}\alpha$ and β , G-CSF, GM-CSF and $\text{TGF}\beta$ (32). These cytokines are important to CD4^+ T cell proliferation, differentiation and function, although, the role of these cytokines in regulating CD4^+ T cell Fc γ RII/III expression is unknown. The role of these cytokines could be directly assayed by examining Fc γ RII/III expression on *in vitro* activated CD4^+ T cells that have been treated with the various cytokines. Their role could also be indirectly assessed through the addition of anti-cytokine mAb to transwell experiments. Furthermore, the variability in the

induction of Fc γ RII/III expression on the CD4⁺ T cells by the subclones could indicate that the subclones are generating different quantities of soluble factors. This hypothesis is supported by the observation that the CH12.LX subclones generate varying levels of secreted cytokines (32). Utilizing ELISA, the cytokine profile and the quantity of cytokines generated by the separate subclones could be determined.

It was also observed that the subclones did not equally allostimulate the CD4⁺ T cells to proliferate on a per cell basis, even though they were derived from the same cell line (21). This observation suggests that the sublines may express varying levels of MHC class II or costimulatory molecules, such as B7, which are necessary for CD4⁺ T cell activation and proliferation (33-36). Therefore, it is also possible that the variation in the levels of Fc γ RII/III expression induced by the different subclones could be attributed to varying levels of surface MHC class II or costimulatory molecules. Since, the level of surface MHC class II, B7, or other costimulatory molecules, can be determined using FACS analysis (37), it would be possible to evaluate the role of these molecules in the induction of CD4⁺ T cell Fc γ RII/III expression. CD4⁺ T cells could be cocultured with the different CH12.LX B cell subclones while being subjected to equal or varying levels of MHC class II or costimulatory molecules.

In conclusion, the data indicate that CH12.LX B cells secrete soluble factor(s) that augment Fc γ RII/III expression on alloactivated CD4⁺ T cells and that Fc γ RII/III expression may not be dependent upon the Ig isotype of the CH12.LX B cell. However, additional investigation is required to further define the role of B cells, Ig and cytokines in the induction and regulation of Fc γ RII/III expression on CD4⁺ T cells.

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Chapter Six**Summary**

FcR are transmembrane glycoproteins that bind the Fc domain of Ig (Ravetch and Kinet, 1991). These receptors are expressed on virtually all haematopoietic cells (Ravetch and Kinet, 1991) and they mediate a variety of immunological functions (Ravetch and Kinet, 1991; Fanger et al., 1989; Lanzavecchia, 1990; Manca et al., 1991; Walker, 1977; Ukkonen et al., 1986; Ishizaka and Ishizaka, 1978; Anegón et al., 1988). However, the expression of these receptors and their function on CD4⁺ T cells is not fully understood. The series of studies, described in the preceding chapters, were conducted to examine CD4⁺ T cell FcR expression and function.

Previous work has demonstrated that activation of CD4⁺ T cells, with antigen-pulsed macrophages or IL-1, induces the generation of IgGBF and IgABF, which participate in the generation of soluble macromolecules (Ernst et al., 1989; Maeba et al., 1988). These macromolecules, known as contrasuppressive factors (CSF), are comprised of IgBF, MHC class II and Ig (Maeba et al., 1988; Paraskevas et al., 1979; Paraskevas et al., 1985). These factors act to augment antigen- and isotype-specific responses in immunologically suppressed systems, *in vivo* (Lee and Paraskevas, 1981) and *in vitro* (Ernst et al., 1989; Maeba et al., 1988). CD8⁺ T cells and T cell lines have also been shown to modulate antigen-specific B cell responses and Ig synthesis, *in vivo* and *in vitro*, and this activity is mediated through the generation of sFcR (Mathur and Lynch, 1986; Daeron et al., 1989; Varin et al., 1989; Fridman et al., 1992; Brunati et al., 1990; Teillaud et al., 1990b). Therefore, I initially investigated whether the CD4⁺ T cell derived IgGBF and IgABF were sFcγRII/III and sFcαR, respectively. Data from these studies showed that the IgBF generated by the CD4⁺

T cells were serologically and functionally indistinguishable from sFcR. Purified sFc γ RII, at concentrations of 0.01 to 0.5 ng/ml, could functionally substitute for the IgGBF in the enhancement of isotype-specific antibody responses *in vitro*.

The enhancing activity of the CSF is mediated directly through a multi-cellular pathway known as the contrasuppressor pathway that blocks T cell mediated suppression of IgG and IgA responses (Ernst et al., 1988; Zettel et al., 1992; Al-Maghazachi et al., 1983; Lee and Paraskevas, 1981; Paraskevas et al., 1985). These factors activate a subset of T cells that adhere to the lectin *Vicia villosa* (Vv) and the Vv adherent T cells block the activity of antigen-specific suppressor T cells (Ts) *in vivo* and *in vitro* (Lee et al., 1985; Zettel et al., 1992; Ernst et al., 1988). Data from this study also demonstrated that the isotype-specific enhancing activity of the IgGBF and IgABF was mediated via the Vv T cells. Since, the sFcR could functionally substitute for the IgBF, the previous observation would indicate that the sFcR are also mediating their isotype-specific enhancing activity through the Vv T cells. It is known that the CSF activate Vv T cells by adhering to the surface of these cells (Paraskevas and Lee, 1981; Lee et al., 1985; Ernst et al., 1988), however, it remains unclear how they mediate their activity upon the Vv T cells. Furthermore, it is not known how the Vv T cells mediate their activity to enhance both isotype- and antigen specific responses. These cells could either act upon the B cells to stimulate antibody responses or make them refractory to the Ts cells. Vv T cells may also directly modulate Ts cell functions. One report indicated that TGF β_1 could directly inhibit Ts cell activity and enhance antibody responses *in vitro*. However, it is unlikely that the Vv T cells are mediating their activity via the

generation of $\text{TGF}\beta_1$, since it did not exhibit isotype-specificity (Zettel et al., 1992).

I also observed that sFc γ RII/III failed to augment IgG anti-SRBC responses at concentrations 10 to 100 ng/ml. It has been reported that sFc γ RII/III, at concentrations of 2 ng/ml to 20 μ g/ml, can inhibit both primary and secondary antibody responses *in vitro* (Varin et al., 1989). This activity of the sFc γ RII/III is mediated directly upon the B cells, inducing a down regulation in the transcription of both the Ig heavy and light chain genes (Daeron et al., 1989; Teillaud et al., 1990a). Therefore, it is possible that the observed loss of IgG enhancing activity, when sFc γ RII/III was used at concentrations of 10 to 100 ng/ml, could be attributed to this mechanism.

Collectively, these results may provide some insight into the regulation of Ig isotype at mucosal surfaces. Mucosal associated lymphoid tissues (MALT) are characterized by predominant IgA responses (Strober, 1990; Mazanec et al., 1993; Weinstein et al., 1991). Moreover, previous work has shown that Peyer's patch CD4⁺ T cells predominately generate IgABF (Ernst et al., 1988) and express surface Fc α R (Char et al., 1991). Therefore based on these observations and the sFcR data, it is possible to hypothesize that *in vivo* Peyer's patch CD4⁺ T cells preferentially secrete sFc α R and thereby selectively induce IgA production. This model fits with the phenomenon of oral tolerance in which IgA antibodies are produced in the MALT while IgG antibody synthesis is suppressed (Tomasi Jr. and Challacombe, 1980; Richman et al., 1981). Reports have demonstrated that T cells, which regulate IgA production, arise within the Peyer's patches following oral administration of antigen at doses that induce oral tolerance (Richman et al., 1981; Suzuki et al., 1986). These

cells control antigen-specific IgA responses and develop in the Peyer's patches within 24 hours of oral antigenic stimulation (Richman et al., 1981; Mattingly, 1983). Therefore, it is possible that following antigenic stimulation, Peyer's patch CD4⁺ T cells release small amounts of sFcαR that promote the induction of IgA by controlling the local antigen-specific Ts cells.

Since the previous data indicated that activation of CD4⁺ T cells induced the generation of sFcγRII/III, I proceeded to investigate FcγR expression on CD4⁺ T cells. In this study it was observed that alloactivation of CD4⁺ T cells induced *de novo* FcγRIIB1 expression. Little is known about the functional role of these receptors on CD4⁺ T cells, however, several studies have investigated the functional role of FcγRIIB1 on B cells. FcγRIIB1, unlike the other FcγR, does not induce cellular activation when crosslinked. Moreover, when crosslinked with the BCR, FcγRIIB1 induces a negative regulatory signal that inhibits Ca²⁺ influx (Wilson et al., 1987; Amigorena et al., 1992a; Bijsterbosch and Klaus, 1985; Choquet et al., 1993) and, IP₃ and DAG production (Bijsterbosch and Klaus, 1985), which ultimately blocks B cell proliferation, differentiation and Ig synthesis (Fridman, 1993; Sidman and Uranue, 1976; Klaus et al., 1984; Phillips and Parker, 1984; Phillips and Parker, 1983).

The BCR and TCR share common ARAM activation motifs within their IC domains (Desiderio, 1992; Keegan and Paul, 1992; Weiss and Littman, 1994; Cambier, 1992; Malissen and Schmitt-Verhulst, 1993). The signal transduction pathways of both receptors have been mapped and these receptors were found to have common signal transduction elements

(Cambier and Ransom, 1987; Fridman, 1993; Ravetch, 1994; Weiss and Littman, 1994; Klausner and Samelson, 1991; Keegan and Paul, 1992; Cambier, 1992; Reth, 1992; Weiss, 1993). Based on these observations and the fact that Fc γ RIIB1 can block B cell proliferation and differentiation, it has been suggested that the functional role of Fc γ RIIB1 on CD4⁺ T cells is also negative (Ravetch, 1994; Daeron et al., 1995; Kwack et al., 1995). Some preliminary publications have indicated that Fc γ RIIB1 can inhibit TCR activation (Kwack et al., 1995; Daeron et al., 1995).

Fc γ RIIB1 and Fc γ RIII expression has been detected in murine thymocytes (Sandor et al., 1994; Rodewald et al., 1992). In addition, an alternative ligand for these two receptors has been identified on the local thymic stromal cells (Sandor et al., 1994). Fc γ RIII expression requires association with the γ subunit for expression (Ra et al., 1989; Kurosaki et al., 1991; Hibbs et al., 1989) and signal transduction (Amigorena et al., 1992b; Letourneur and Klausner, 1991; Eiseman and Bolen, 1992; Wirthmueller et al., 1992) and this subunit can functionally substitute for the ζ subunit of the CD3 complex (Ohno et al., 1993; Rodewald et al., 1991). Furthermore, several surface markers, such as CD2 and Thy-1, which require the TCR-CD3 complex to mediate their signals (Frank et al., 1990; Moigeon et al., 1992) are expressed prior to TCR-CD3 expression (Rodewald et al., 1992). Therefore, it is possible that the Fc γ RIII may functionally substitute for the TCR-CD3 complex during early thymocyte differentiation and may be involved in regulating T cell differentiation. This hypothesis is supported by the observation that blocking of the Fc γ RII/III-ligand binding *in vitro* altered T cell differentiation (Sandor et al., 1994). However, in the γ subunit knockout

mouse T cells develop and function normally (Takai et al., 1994). This observation implies that the Fc γ RIIB1, but not Fc γ RIII, may be involved in regulating T cell differentiation.

Fc γ RIIB1 expression on CD4⁺ T cells may provide a mechanism for regulating these cells and immune responses. Fc γ RIIB1 expression on B cells is thought to provide a feedback mechanism for controlling B cell activation through cognate antibody, which would allow for the maintenance of Ig homeostasis after antigen stimulation (Fridman, 1993; Ravetch, 1994). Crosslinking of the BCR and Fc γ RIIB1, *in vivo*, could only occur if the IgG bound to the Fc γ RIIB1 was specific for the antigen bound to the BCR (Dickler and Kubicek, 1981; Phillips and Parker, 1983). Fc γ RIIB1 expression on CD4⁺ and CD8⁺ T cells may also represent a negative feedback mechanism for B cells to control T cells and ultimately regulate the immune response. Crosslinking could occur in the same manner as with the B cells, however, the IgG bound to the Fc γ RIIB1 would recognize the antigen present in the TCR. Crosslinking could also occur during cognate interactions with activated B cells. Reports have indicated that T cells and B cells can form cognate interactions via other FcR and surface Ig (BCR) (Teeraratkul and Lynch, 1991). It has been also suggested that only Th2 type CD4⁺ T cells express FcR (Sandor et al., 1990a), therefore the Fc γ RIIB1 pathway could represent a mechanism for regulating Th2 type responses. The effect of Fc γ RIIB1 and BCR or TCR crosslinking on cytokine production has not been evaluated. However, it has been reported that the crosslinking of Fc ϵ RI, which contains an ARAM activation motif, and Fc γ RIIB1 could inhibit the release of stored serotonin and TNF α production (Daeron et al., 1995).

Cellular activation, via TcR, has been demonstrated to induce FcR expression on

some CD4⁺ cell lines (Sandor et al., 1990a; Char et al., 1991) and on freshly isolated CD8⁺ T cells (Klausner and Samelson, 1991) and CD8⁺ T cell lines (Briere et al., 1988). In my studies, I also observed that MHC class II restricted antigen presentation could induce FcγRIIB1 expression, although, direct TCR ligation was not sufficient for the induction of FcγRIIB1 expression on CD4⁺ T cells. Furthermore, I observed that the IgG2b⁺ CH12.LX B cells were generating a soluble factor(s) that promoted or enhanced FcγRIIB1 expression on alloactivated CD4⁺ T cells. Previous publications have indicated that elevated levels of Ig, either *in vivo* or *in vitro*, could induce FcR expression on CD8⁺ T cells. In my studies, soluble IgG2b failed to induce FcγRIIB1 expression on alloactivated CD4⁺ T cells. The inability of the soluble IgG2b to induce FcγRIIB1 expression in this study could be attributed to the fact that physiological concentrations of IgG2b were used, while in the reports, the authors used supermaximal doses of Ig (Hoover et al., 1981; Mathur et al., 1986; Adachi et al., 1983; Hoover and Lynch, 1980; Mathur and Lynch, 1986; Coico et al., 1985).. Moreover, the *in vivo* induction of FcR expression on CD8⁺ T cells occurred under conditions where the concentration of serum Ig reached pathological levels (Hoover et al., 1981; Adachi et al., 1983; Mathur et al., 1986). A previous report also documented that elevated levels of IgA could downregulate FcγRIIB1 expression on CD8⁺ T cells (Yodoi et al., 1983). Therefore, the inability of the soluble IgG2b to induce FcγRIIB1 expression could also be attributable to the soluble IgA present during the experiment (Yodoi et al., 1983). The role of Ig in the regulation of FcγRIIB1 expression on CD4⁺ T cells is not fully understood and remains to be defined.

Cytokines have also been shown to regulate FcR expression in several cell lineages (Yodoi et al., 1981; Fridman et al., 1980; Yodoi et al., 1983; Schreiber et al., 1990; van de Winkel et al., 1990; Yodoi et al., 1983; Conrad et al., 1987; Swenson et al., 1993). The CH12.LX B cell line is a CD5⁺ (B1) B cell lymphoma that constitutively secretes Ig and a variety of cytokines, which can activate and promote T cell differentiation (Arnold et al., 1983; Whitmore et al., 1991; Louie et al., 1993; O'Garra and Howard, 1992). The CH12.LX B cell line reportedly generates the cytokines IL-3, 4, 6 and 10, TNF α and β , GM-CSF, G-CSF and TGF β (Louie et al., 1993; O'Garra and Howard, 1992). Moreover, the level of cytokine production varies between the different subclones (O'Garra and Howard, 1992). Currently, there is no data that examines the role of these cytokines or other cytokines in the regulation of Fc γ RIIB1 expression on CD4⁺ T cells and further investigation is required. Nevertheless, data from my study indicate that B cell derived factors, possibly cytokines, are involved in regulating Fc γ RIIB1 expression on activated CD4⁺ T cells.

I also observed that Fc γ RIIB1 expression on CD4⁺ T cells occurred within 24 hours of alloactivation and prior to DNA synthesis. Similar kinetics of expression has also been observed in activated B cells, in which Fc γ RIIB1 expression is upregulated early during the G₁ phase, prior to DNA synthesis (Amigorena et al., 1989). This observation suggests that the mechanisms regulating the induction of Fc γ RIIB1 expression on activated CD4⁺ T cells and B cells may be similar. Several regulatory sequences, including an NF κ B-like binding site, have been identified either 5' or within the Fc γ RII gene, however, their role in regulating Fc γ RII expression is not known (Hogarth et al., 1991; Bonnerot et al., 1988). Interestingly,

both B cells and macrophages constitutively express high levels of FcγRII (Ravetch and Kinet, 1991; Titus et al., 1984; Weinshank et al., 1988; Amigorena et al., 1989; Grilli et al., 1993) and this could be attributed to their constitutive generation of the active form of NFκB (Sen and Baltimore, 1986a; Sen and Baltimore, 1986b; Kieran et al., 1990; Grilli et al., 1993). Furthermore, activation of T cells with mitogens (Sen and Baltimore, 1986b; Bohnlein et al., 1988; Grilli et al., 1993) or anti-CD3 (Tong-Starksen et al., 1989; Grilli et al., 1993) stimulates the generation of active NFκB transcription factors (Ghosh and Baltimore, 1990; Grilli et al., 1993), which promotes the induction of several genes (Kieran et al., 1990; Lenardo and Baltimore, 1989; Grilli et al., 1993; Bohnlein et al., 1988), including the early expression of the IL-2R (Lenardo and Baltimore, 1989; Grilli et al., 1993; Bohnlein et al., 1988). Therefore, it is possible that early induction of FcγRIIB1 expression on the alloactivated CD4⁺ T cells is mediated through NFκB transcription factors.

Data from this study clearly demonstrate that alloactivation of CD4⁺ T cells induces early *de novo* expression of FcγRIIB1 and its expression is influenced by soluble factor(s) secreted by CH12.LX B cells. In addition, activation of CD4⁺ T cells induces the generation of sFcγRII/III and sFcαR that enhance antibody responses, *in vivo* and *in vitro*, in an isotype-specific manner. These observations suggest that FcR on CD4⁺ T cells may mediate a broad range of immunoregulatory functions and that CD4⁺ T cell FcR expression may represent novel mechanisms for regulating immune responses. FcγRIIB1 expression on activated T cells may provide a negative feedback mechanism for controlling activated CD4⁺ and CD8⁺ T cells, while the generation of sFcR may represent a mechanism for inducing antigen- and

isotype-specific antibody responses in immunologically suppressed environments, such as the gut.

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