Modulation of CD4$^+$ T cell Responses by CD59a

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A thesis submitted to Cardiff University in Candidature for the Degree of
Doctor of Philosophy

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UMI Number: U584800

UMI

Dissertation Publishing

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"Science is the art of creating suitable illusions, which the fool enjoys or argues against, but the wise man enjoys for their beauty or ingenuity, without being blind to the fact that they are human veils and curtains concealing the abysmal darkness of the unknowable"

Carl Jung

To my mum for being a constant inspiration for my journey in life

Acknowledgements

Abstract

CD59 is a GPI-anchored protein found in membrane microdomains known as lipid rafts. It is a complement regulator protein, which blocks the formation of the membrane attack complex by inhibiting binding of C9 to the C5-8 complex. Human CD59 has also been described as a co-stimulator of T cell activation. The aim of this project was to analyze the role of CD59 on T cell activation *in vivo*. For this purpose, anti-viral CD4$^+$ T cell responses were analyzed in mice deficient in the mouse analogue of CD59; CD59a. Infection with recombinant vaccinia virus (rVV) and influenza virus, resulted in stronger virus-specific CD4$^+$ T cell responses in Cd59a -/- mice compared to WT mice. This effect, which indicates that CD59a downmodulates antigen-specific T cell activity, was found to be complement independent. Experiments were performed to investigate the effect of CD59 expression on human CD4$^+$ T cells. Blocking CD59 increased proliferation of the cells *in vitro* indicating that CD59 might similarly downmodulate human CD4$^+$ T cell activity. Using mouse T cells, mechanisms underlying the effect of CD59a on CD4$^+$ T cell activity were investigated. Results of these studies indicated that downmodulation of T cell activity through CD59a requires engagement of CD59a with a ligand expressed on APCs.

To assess the biological consequences of CD59a deficiency, the extent of immunopathology induced following infection with influenza virus was compared in WT and Cd59a-/- mice. Immunopathology was exacerbated in Cd59a-/- mice, correlating with increased numbers of neutrophils and CD4$^+$ T cells in infected lungs. When complement was inhibited, lung-infiltrating neutrophils in Cd59a-/- mice were much reduced while numbers of infiltrating-CD4$^+$ T cell remained unchanged. These results demonstrate that CD59a is more than a regulator of complement but can in fact, alter both the innate and adaptive immune responses using both complement dependent and independent mechanisms.

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Abbreviations

Ab	antibody
AIA	antigen-induced arthritis
AICD	activation induced cell death
ANTN	accelerated nephritic nephritis
APC	antigen presenting cell
B-ME	β-mercaptoethanol
CFD	complement fixation diluent
CFSE	carboxyfluorescein diacetate succinimidyl ester
CHS	contact hypersensitivity
C1inh	C1 inhibitor
CReg	complement regulatory proteins
Crry	complement receptor related protein-y
CR1	complement receptor 1
CVS	cobra vena factor
DC	dendritic cell
DP	double positive
E	erythrocytes
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EEV	extracellular enveloped virus
EGF	epidermal growth factor
fH	factor H
GEMs	glycosphingolipid-enriched microdomains
GP	glycoprotein
GPI	glycosyl phosphatidylinositol
HA	haemagglutinin
H&E	hematoxylin and eosin
HIV	human immunodeficiency virus
iDC	immature dendritic cell
IEV	intracellular enveloped virus
ILT3	immunoglobulin-like transcript 3
IMV	intracellular mature virus
i.n.	intra-nasally
iNOS	nitric oxide synthase
i.p.	intra-peritoneal
IRI	ischemia-reperfusion injury
IS	immunological synapse
i.v.	intra-venous
LAT	linker for activation of T cells
LCMV	lymphocytic choriomeningitis virus
M	matrix proteins
Mab	monoclonal antibody
MAC	membrane-attack complex
MASP	MBL-associated serine proteases

MBL	mannan binding lectin
MCP	membrane co-factor protein
mDC	mature dendritic cell
MF	mean fluoresence
MVA	vaccinia virus Ankara
MW	molecular weight
NA	neuraminidase
ON	overnight
PAG	phosphoprotein associated with glycosphingolipid-enriched microdomains
PBMCs	peripheral blood mononuclear cells
PIG-A	phosphatidylinositol glycan-class A
PNH	paroxysmal nocturnal haemoglobinuria
RCA	regulators of complement activation
rCD59	recombinant CD59
RNP	ribonucleoprotein complex
RT	room temperature
rVV	recombinant vaccinia virus
SCR	short consensus repeat
sCR1	soluble CR1
SERPINs	serine protease inhibitors
SI	stimulation index
siRNA	small interfering RNA
SMAC	supramolecular activation cluster
Tc	cytotoxic T cell
Treg	regulatory T cell
WT	wild-type
VSV	vesicular stomatatis virus
VV	vaccinia virus

Chapter 1- Introduction

1.1. The Complement System

Complement is an arm of the innate immune system, providing a rapid and efficient means for opsonising or killing pathogens and triggering inflammation. The complement system is made up of a large number of distinct plasma proteins produced mainly by hepatocytes but also by different tissues and cells including macrophages and gut epithelial cells. These proteins are activated by a variety of agents and their activation proceeds in a cascade fashion leading to lysis. In this way, the activation of a small number of complement proteins at the start of the pathway is highly amplified by successive enzymatic reaction, resulting in the rapid generation of a large complement response.

Complement activation can be divided into three pathways classical, alternative and lectin pathway. These pathways depend on different molecules for their initiation, but they converge in the last steps of the cascade (membrane-attack complex; MAC).

The classical pathway is activated by antibody opsonization of pathogens (binding of antibodies to specific epitopes of surface proteins) and is therefore linked to the adaptive immune system. This pathway is initiated by the binding of C1q to a specific part of the antibody molecule. C1q is part of the C1 complex, which comprises a single C1q molecule bound to two molecules each of C1r and C1s (Schumaker *et al.*, 1976). Upon C1q binding to an antibody-antigen complex, the

homologous C1r and C1s subcomponents are converted into catalytically active

species to generate an active serine protease triggering the first step of the classical

pathway of complement activation (Rivas *et al.*, 1994). Then, C1s cleaves C4 to

produce C4b, which binds covalently to the surface of the pathogen (Muller-Eberhard

and Lepow, 1965). The formation of an active C4b site creates the binding site for C2.

Cleavage of C2 by C1s results in the formation of a large fragment, C2a, that becomes

firmly associated with C4b and a smaller fragment C2b. The complex, C4b2a, remains

on the surface of the pathogen as the C3 convertase of the classical pathway (Figure

1.1A).

The lectin pathway is initiated by a component which is a serum lectin, Mannan

Binding Lectin, MBL, which binds pathogen surface molecules containing mannose

residues. MBL in the lectin pathway is equivalent to C1q in the classical complement

pathway (Lu *et al.*, 1990). When the MBL complex binds to a pathogen surface,

MASP-1 and MASP-2 (MBL-associated serine proteases; equivalent to C1r and C1s of

the classical pathway) are activated to cleave C4 and C2 and form C4b2a, the C3

convertase (Thiel *et al.*, 1997; Weis *et al.*, 1992)(Figure 1.1A). In contrast, the

alternative pathway is initiated by spontaneous hydrolysis of C3. C3 has been shown to

contain a thioester bond which reacts with almost anything that exposes -OH or -NH2

groups, by spontaneously "ticking over"(Hostetter *et al.*, 1982; Lachmann and Hughes-

Jones, 1984; Pangburn and Muller-Eberhard, 1980). In this arm of complement,

spontaneous low-level cleavage of C3 converts inactive native C3 to a functionally

active C3b-like molecule referred to as C3(H2O) which, in the presence of Mg^{2+}, binds

the plasma protein factor B, that is subsequently cleaved by factor D, leading to the

generation of a distinct C3 convertase designated C3b,Bb (Fearon and Austen, 1975a).

The resulting C3 convertase is stabilized by the binding of properdin (P) that increases

the half-life of this convertase (Fearon and Austen, 1975b)(Figure 1.1B). All three

pathways converge at the enzyme activity of the C3 convertase. The function of the

initial C3 convertase is to produce nascent C3b and to deposit C3b on the surface of

surrounding particles. Alternatively C3b can become bound to C3 convertase resulting

in formation of C5 convertase (C4b2a3b for classical pathway or C3bBbC3b for

alternative pathway) which cleaves C5 into C5a and C5b, the first known as the most

potent of the anaphylatoxins (Daha et al., 1976; Hugli and Muller-Eberhard, 1978;

Medicus et al., 1976).

One of the important effects of complement activation is the assembly of the

terminal components of complement to form the MAC. The MAC constitutes a

supramolecular organisation where one molecule each of C5b, C6, C7, and C8 form

C5b-8 that catalyzes the polymerization of C9 to form the MAC (Tschopp et al., 1982).

This structure inserts into target membranes and causes cell lysis (Hu et al., 1981;

Podack et al., 1982) (Figure 1.1C).

Activation of the complement cascade leads to the fragmentation of C3, C4

and C5 releasing low-molecular-weight peptides, C3a, C4a, and C5a with

anaphylatoxin activity (Bokisch et al., 1969; Gorski et al., 1979). Anaphylatoxins are

biologically active peptides that are defined functionally by their actions on small

blood vessels, smooth muscle, mast cells, and peripheral blood leukocytes. They bind

to specific receptors and induce the release of vasoactive amines such as histamine

from mast cells and basophils, and lysosomal enzymes from granulocytes (particularly

C3a and C5a) (Johnson *et al.*, 1975; Schulman *et al.*, 1988). C5a is the most active

followed by C3a and finally C4a with approximately 500-fold less activity than C3a

(Gorski *et al.*, 1981). In addition, complement activation can significantly enhance the

immune response by antigen opsonization. Antigen bound C3d, iC3b and C4b can be

recognized by receptors on a variety of cells such as macrophages, monocytes, B

lymphocytes, neutrophils, erythrocytes and platelets (Schreiber, 1984). Therefore, C3b

and C4b serve as a bridge between the antigen and the phagocytic/responder cell.

1.2. Complement Regulation

Due to the propensity of the system to tick over, to undergo rapid and extensive

amplification once triggered and to damage bystander cells because active components

'drift' away from activating sites, self cells have evolved an armoury of proteins which

protect membranes from damage by homologous complement. Some of these are

soluble, plasma proteins, whereas others are expressed on cell membranes.

Complement regulatory proteins (CReg) serve to protect host tissue against the

potentially destructive effects of complement. The CReg comprise three structurally

and functionally distinct families of proteins which collectively control the classical,

alternative and lectin C pathways: the serine protease inhibitors (SERPINs), the

"regulators of complement activation" (RCA) family, and CD59. SERPINs include the

C1 inhibitor (C1inh). C1inh binds the active enzyme C1r:C1s, and causes it to

dissociate from C1q, which remains bound to the pathogen. In this way, C1inh limits

the time during which active C1s is able to cleave C4 and C2.

The RCA family includes factor H (fH), C4b binding protein (C4bp), decay accelerating factor (DAF; CD55), membrane co-factor protein (MCP; CD46) and complement receptor 1 (CR1; CD35) (Holers and Kotzin, 1985; Hourcade *et al.*, 1989). In common with all members of the RCA family, they contain a structural module comprising approximately 60 amino acids, termed the short consensus repeat (SCR) (Barlow *et al.*, 1991). CD46 and CD55 each have four of these modules, the C3b-binding and functional activities of the proteins reside in this region (Liszewski *et al.*, 1991; Nicholson-Weller and Wang, 1994; Reid and Day, 1989) (Figure 1.2). On cell membranes, CD55 regulates complement by binding to the C3 convertases, C4b2a (classical pathway) and C3bBb (alternative pathway), accelerating their decay (decay accelerating activity) thereby interfering with both the classical and alternative arms of the complement cascade (Figure 1D) (Fujita *et al.*, 1987; Medof *et al.*, 1984). CD46 binds to either C3b or C4b enabling a plasma serine protease, factor I (fI), to proteolytically cleave the active component and irreversibly inactivate the enzymes (co-factor activity) (Liszewski and Atkinson, 1996; Seya and Nagasawa, 1981). CD55 is also expressed in rodents where it performs a similar CReg function (Harris *et al.*, 1999; Hinchliffe *et al.*, 1998; Spicer *et al.*, 1995); in contrast, CD46 is only expressed in rodent testis and is not present in other tissues or circulating cells (Miwa *et al.*, 1998; Mizuno *et al.*, 2005). Instead, rodents have another membrane CReg, complement receptor related protein-y (Crry), which is expressed on blood cells and in most tissues (Funabashi K, 1994; Li *et al.*, 1993). Crry is also built from a 'string' of SCR domains and can perform the functions of both CD55 and CD46 i.e. it promotes decay of the convertase enzymes and acts as a cofactor for factor I-mediated cleavage of C3b and C4b (Kim *et al.*, 1995; Quigg *et al.*, 1995). CR1 exhibits decay accelerating activity and co-factor activity (Iida and Nussenzweig, 1983)(Figure 1.1D).

A

Classical/lectin pathway

C4

C2

Activated C1
(or MBL)

C3

C3a

C3b

C5

C5a

C4b2a

C4b2a3b

C5b

Cell

B

Alternative pathway

Bb

fD

fB

C3

Proteases/
Fluid phase
Convertase
enzymes

C3a

C3a

fB

C5

C5a

C3b

C5b

C3bB

C3bBb

fD

C3bBbC3b

Cell

C

Terminal pathway

C7

C8

C9

C6

C5b

C5b67

C5b678

MAC

D

CD55

'Decay acceleration'

C4b2a

C1/MBL

CD46

Factor I

'Cofactor activity'

C3b   iC3b

C3b

Classical/
Lectin
Pathway

Alternative
Pathway

C5b

Terminal
Pathway

CD59

MAC

21

Figure 1.1. Complement Activation and Regulation.

Diagram of the complement activation pathways. (A) The classical pathway is activated by antibody. Binding of C1 to antibody leads to cleavage of C4 and deposition of C4b on the membrane. C2 associates with C4b and is cleaved and activated by C1 to form the C3 convertase. As with the alternative pathway, multiple molecules of C3b are cleaved to form the C5 convertase. The lectin pathway is identical to the classical pathway except that it is initiated by mannan-binding lectin (MBL) binding to carbohydrate moieties, this activates the MASPs (MBL-associated serine proteases) which cleave C4 and C2 in a manner similar to C1. (B) The alternative pathway ticks over spontaneously in plasma resulting in C3b-like molecules or C3b which can deposit on cell membranes. C3b binds factor B which is cleaved and activated by fD to form the C3 convertase. C3b can associate with the initiating enzyme to form the C5 convertase. The C5 convertase cleaves multiple molecules of C5 to C5a and C5b, initiating the terminal pathway. (C) The activation pathways converge at the point of C5 cleavage. C5b remains associated with the C5 convertase where it binds C6 and C7. The C5b67 complex releases from the convertase and binds loosely to the membrane, it rapidly binds C8 forming the C5b678 complex. C9 binds to the complex and poymerises to form the MAC. (D) Regulation of the activation and terminal pathways is brought about through the actions of the membrane associated CReg: CD46, CD55 and CD59. CD55 has decay accelerating activity and acts by binding the C3 and C5 convertases of all activation pathways (the classical pathway C3 convertase is illustrated here). Binding of CD55 accelerates the decay of the enzyme by promoting release of the enzyme subunit Bb or C2a. By dissociating the enzymes it prevents amplification of the cascade by further C3 cleavage and enzyme formation. CD46 has cofactor activity, it binds C3b or C4b and enables a plasma serine protease, factor I, to cleave and inactivate the protein. CD59 regulates the terminal pathway by binding the C5b678 complex preventing C9 polymerisation and pore formation.
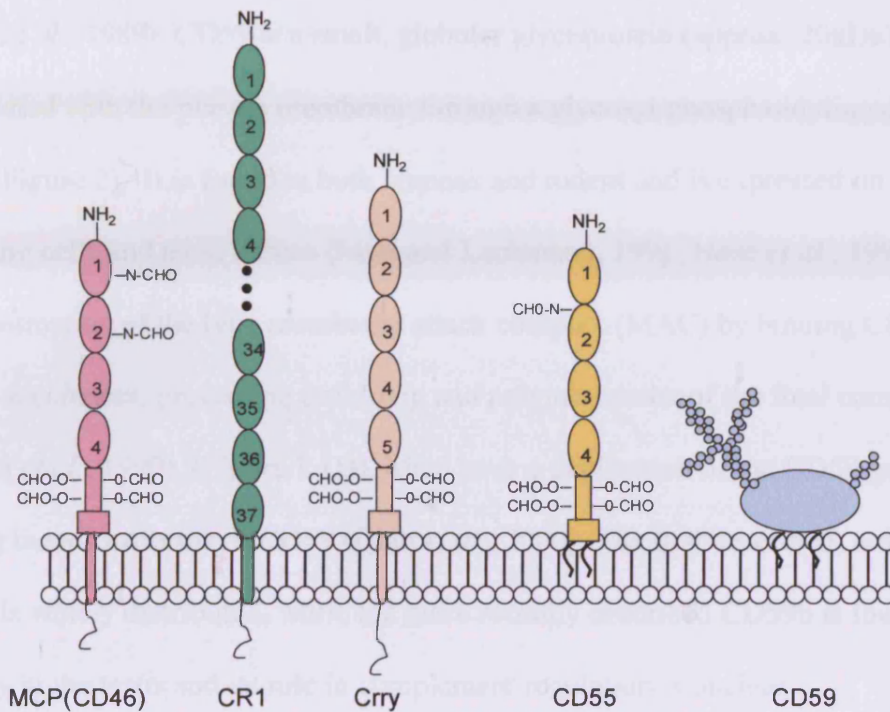
Figure 1.2. Structure and Function of CReg
CD35 (CR1), CD46, CD55 and Crry are members of the RCA family and contain a structural module termed the short consensus repeat (SCR). Crry is only present in rodents and is formed of 5 SCR domains in mice and 7 SCR domains in rat. CD59 is a small highly glycosylated molecule and, like CD55, is attached to the membrane via a GPI-anchor.

Whilst several proteins collaborate to regulate the activation arms of the complement cascade, only one membrane CReg, CD59, inhibits the terminal pathway (Davies *et al.*, 1989). CD59 is a small, globular glycoprotein (approx. 20kDa), which is associated with the plasma membrane through a glycosyl phosphatidylinositol (GPI) anchor (Figure 2). It is found in both humans and rodent and is expressed on all circulating cells and most tissues (Meri and Lachmann, 1991; Nose *et al.*, 1990). It blocks formation of the lytic membrane attack complex (MAC) by binding C8 within the C5b-8 complex, preventing unfolding and polymerisation of the final component, C9 (Meri *et al.*, 1990). (Figure 1.1D). Mice have a duplication of the CD59 gene, resulting in two isoforms of CD59 (Qian *et al.*, 2000). The first described, now termed CD59a, is widely distributed, while the more recently described CD59b is found primarily in the testis and its role in complement regulation is unclear (Baalasubramanian *et al.*, 2004; Harris *et al.*, 2003).

The importance of CReg in protecting host cells from complement attack is illustrated by the consequences of CReg deficiency in humans. Patients with Paroxysmal Nocturnal Haemoglobinuria (PNH) exhibit a deficiency in CD55 and CD59 on clonal populations of erythrocytes, platelets and other circulating cells as a result of stem cell mutations in the phosphatidylinositol glycan-class A (PIG-A) gene essential for GPI anchor synthesis (Takeda J, 1993). The disease is characterized by complement-mediated haemolysis, venous thrombosis, and bone marrow failure (Takeda J, 1993). In rodents, the protective role of CReg is best illustrated by deficiency of Crry. First, Crry is crucial for embryonic development since its deficiency result in embryonic lethality (Xu *et al.*, 2000); second, Crry deficient

erythrocytes are subject to rapid elimination from the circulation in normal mice despite expression of CD55 and CD59, demonstrating that Crry is indispensable for protecting mouse erythrocytes from spontaneous complement attack (Molina *et al.*, 2002)

1.3. Alternative Roles for CReg

A growing body of evidence indicates that CReg are not just regulators of complement and there is an ever-expanding and diverse list of roles that these proteins play. Roles include acting as pathogen receptors, influencing neutrophil migration and promoting cell proliferation, signal transduction and LPS-induced activation of various cell types (Cattaneo, 2004; Davis *et al.*, 1988b; Harris and Morgan, 2004; Heine H, 2003; Lawrence *et al.*, 2003; Morgan *et al.*, 1993; Yamamoto *et al.*, 2003). There is also striking evidence that CReg, most notably CD46, play a part in fertilisation and reproduction (Kitamura *et al.*, 1997; Riley-Vargas *et al.*, 2004). Recently, much attention has focussed on the effect of CReg on the activation of antigen-specific T cell responses. These observations, summarised below, imply that CReg, sometimes in collaboration with the complement system, play key roles in regulating the adaptive immune system.

1.3.1. Cross-Linking CReg on T Cells Triggers Cell Activation

Membrane CReg have been implicated in T cell activation almost since their discovery. Several groups demonstrated in early papers that antibody cross-linking of either CD55 or CD59 on human lymphocytes in association with stimulation through the T cell receptor or with phorbol esters, induced re-organization of the cytoskeleton

(Harder and Simons, 1999; Kammer, 1988), cell proliferation (Davis *et al.*, 1988a; Korty *et al.*, 1991), enhanced tyrosine phosphorylation through p56^{lck} and calcium mobilisation (Stefanova *et al.*, 1991b; Stefanova, 1991; Tosello *et al.*, 1998). More recently, studies have shown that antibody ligation of Crry in rodent or CD46 in human promotes tyrosine phosphorylation and cell proliferation of primary T cells stimulated with CD3-specific antibodies (Antic Stankovic *et al.*, 2004 ; Arsenovic-Ranin *et al.*, 2000; Wang *et al.*, 2000; Zaffran *et al.*, 2001). Both Crry and CD46 are transmembrane proteins and, upon ligation with antibody, signals may be transmitted directly via their cytoplasmic tails. Antibody cross-linking of CD46 on primary human T cells induced activation of Vav and Rac, phosphorylation of the adaptor proteins p120^{CBL} and LAT, and phosphorylation of extracellular signal-related kinase mitogen-activated protein kinase (Erk MAPK) (Astier *et al.*, 2000; Zaffran *et al.*, 2001). Collectively, these observations, all derived from antibody cross-linking studies have prompted more detailed analyses of the effect of individual CReg ligated by natural ligands on T cell activity. These are discussed in detail below.

1.3.2. Roles of CD46 in T Cell Function

Several recent publications and reviews have highlighted a role for CD46 in modulation of T cell activity (Kemper *et al.*, 2005; Riley-Vargas *et al.*, 2004). CD46 is expressed in most human cells as various isoforms derived from alternative splicing of one gene (Post *et al.*, 1991). The isoforms differ in the length of the membrane-proximal Ser-Thr rich region, the extent of glycosylation and in the cytoplasmic tail. Two different tails are utilised, termed CYT-1 and CYT-2. Both contain signalling motifs and associate with intracellular kinases (Wang *et al.*, 2000; Wong *et al.*, 1997b).

Interactions between CD46 and its ligands or antibodies induce signalling events that differ according to the cytoplasmic tail. An early *in vitro* study by Wang *et al.* suggested that CYT-2 was tyrosine phosphorylated by the src kinase lck upon cross-linking on Jurkat cells expressing both forms of CD46 (Wang *et al.*, 2000). Subsequently, an elegant study by Marie and colleagues utilising transgenic mice expressing human CD46 with either CYT-1 or CYT-2, showed that cross-linking the different cytoplasmic tails *in vivo* could have dramatically different effects on inflammatory responses in a contact hypersensitivity (CHS) model (Marie *et al.*, 2002). Just prior to sensitisation with DNFB, CD46 was cross-linked *in vivo* using recombinant vesicular stomatatis virus (VSV) expressing the measles virus hemagglutinin, a natural CD46 ligand. Inflammatory responses were monitored following a second challenge with DNFB some days later. CD4$^+$ T cells expressing the CYT-1 isoform showed strong proliferative responses with increased IL-10 production resulting in decreased generation of specific CD8$^+$ T cells and a weak contact hypersensitivity reaction. In contrast, engagement of the CYT-2 isoform on CD4$^+$ T cells resulted in decreased cell proliferation and IL-10 production, strong CD8$^+$ T cell responses and increased inflammation. These results show that the effects of CD46 ligation are dictated by the isoform involved. Kemper *et al.* later demonstrated an immunomodulatory effect of CD46 on human T cells (Kemper *et al.*, 2003). Here, simultaneous engagement of CD4$^+$ T cells with CD3 and CD46-specific antibodies in the presence of IL-2 triggered the induction of T cells exhibiting the characteristics of T regulatory 1-type cells (Tr1 Cells). These cells secreted high amounts of IL-10 and inhibited the proliferation of conventional CD4$^+$ T cells. Human T cells express both CD46 isoforms but the contribution of the different cytoplasmic tails is unexplored in this work. Later studies revealed that CD46-induced Tr1 cells expressed high levels of

granzyme B and perforin and were capable of killing autologous targets cells (Grossman *et al.*, 2004). Collectively, these observations have led to the suggestion that these Tr1 cells may impinge upon other immune responses either through cell lysis or through the immunosuppressive effects of IL-10. Importantly, these and other studies also showed that triggering with a physiologically relevant ligand, such as C3b dimers or pathogens, induced the same phenotype (Kemper *et al.*, 2003; Price *et al.*, 2005).

1.3.3. Crry as a Modulator of T Cells in Rodents

As noted above, CD46 expression in rodents is restricted to testis and its complement regulatory function is taken over by a broadly expressed rodent-specific CReg, Crry. Like CD46, Crry may also impinge upon T cell activation. Experiments performed by Fernandez-Centeno and colleagues demonstrated that concurrent antibody ligation of Crry and CD3 promoted activation of mouse CD4$^+$ T cells and skewed the CD4$^+$ T cell response towards production of the Th2 cytokine, IL-4, rather than IFNγ (Th1 cytokine) (Fernandez-Centeno *et al.*, 2000). Arsenovic-Ranin *et al.* showed that co-incubation of rat thymocytes with Crry- and CD3-specific antibodies enhanced thymocyte proliferation, IL-2R expression and IL-2 release (Arsenovic-Ranin *et al.*, 2000). No studies utilising natural ligands to cross-link Crry have been reported and, because deficiency of Crry results in embryonic lethality, it has not been possible to study the influence of Crry on T cell responses using Crry-deficient animals. Nevertheless, the data obtained thus far indicate that engagement of Crry, like CD46 in humans, may downmodulate T cell responses by inducing production of Th2 cytokines.

1.3.4. Roles of CD55 and CD59 in Human T Cell Activation

CD55 and CD59 are GPI-anchored glycoproteins expressed in all circulating cells and most tissues. As discussed above, antibody cross-linking of either CD55 or CD59 influences T cell activation despite the fact that these proteins lack a cytoplasmic domain. Like other GPI anchored proteins, CD55 and CD59 are found predominantly in membrane microdomains called glycosphingolipid-enriched microdomains (GEMs) or lipid rafts. Lipid rafts are structures within the plasma membrane enriched in cholesterol and containing palmitoylated transmembrane adaptor proteins such as LAT (linker for activation of T cells) and PAG (phosphoprotein associated with glycosphingolipid-enriched microdomains), and a multitude of intracellular signalling molecules including Src family kinases and G-proteins (Rodgers, 2005; Viola *et al.*, 1999). The signalling capacity of GPI-anchored proteins requires interaction with these raft-associated signalling molecules since disruption of lipid rafts causes loss of signalling capacity (Stefanova *et al.*, 1991a). Since antibody cross-linking is likely to induce a different type of response to engagement with a natural ligand, these types of experiments do not indicate that CD55 and/or CD59 modulate T cell activation under physiological conditions. Signalling through CD55 following binding of complement components or complexes has yet to be demonstrated; however, ligation of CD55 on intestinal epithelial cells by *E. coli* adhesin has been shown to result in dramatic cytoskeletal F-actin rearrangements accompanied by recruitment of signal transduction molecules, indicating that a natural ligand can also mediate an effect on the cell through CD55 (Peiffer *et al.*, 1998). For CD59, the natural complement-derived ligand is the forming MAC. As well as its lytic activity, it has been reported that MAC deposition can result in stimulation of IL-1 production and DNA synthesis in glomerular mesangial cells and to initiate Ca^{2+} flux and stimulate procoagulant activity

of platelets (Couser *et al.*, 2001; Lovett *et al.*, 1987; Sims *et al.*, 1988). No relevant non-complement ligands for CD59 have yet been described.

1.3.5. Studies of CD55 Utilising Knockout Mice

Two genes for CD55 have been characterised in mice (named Daf1 and Daf2). Daf1 is GPI-anchored, widely distributed and is the human homologue of CD55, whereas Daf2 lacks a GPI anchor, possesses a transmembrane domain and its expression is restricted to testis and spleen (Lin *et al.*, 2001; Spicer *et al.*, 1995). Mice deficient in Daf1 have been engineered and used to examine the influence of these proteins on T cell activity *in vitro* and *in vivo*.

Two recent studies describe T cell responses in CD55-deficient (Daf1-/-) mice. The first study by Liu *et al.* showed that recall responses measured using spleen cells from mice immunised with OVA or an MHC class II restricted MOG-derived peptide were more vigorous in Daf1-/- mice compared to control mice (Liu *et al.*, 2005). T cells from the Daf1-/- mice produced more IFNγ and IL-2 upon antigen re-stimulation than their wild-type (WT) counterparts, whereas production of the immunosuppressive cytokine IL-10 was reduced. This hyper-responsiveness of T cells from the Daf1-/- mice was due to lack of CD55 on the T cells themselves since antigen presenting cells (APCs) purified from Daf1-/- mice stimulated T cells to produce IFNγ to the same degree as those purified from Daf1+/+ mice. The more vigorous response observed in Daf1-/- T cells was not due to a global increase in sensitivity of the T cells to antigen stimulation since no difference was observed in the response of Daf1-/- and Daf1+/+ T cells to stimulation with superantigen. A key finding was that the enhanced production

of IL-2 and IFNγ by Daf1-/- T cells after re-stimulation with antigen was largely dependent upon a functional complement system since the effect was lost when Daf1-/- mice were back-crossed onto C3-deficient (C3-/-) mice in which complement activity is effectively abolished. A residual, complement-independent effect of Daf1 deficiency on T cell function was suggested by the observation that T cell responses were more vigorous in C3-/-Daf1-/- mice compared to C3-/-Daf1+/+ mice. The complement-independent effect was minor and unlikely to be of physiological relevance, as shown by studies in a T cell-driven experimental autoimmune encephalomyelitis (EAE) model. Here disease was exacerbated in Daf1-/- mice, but not in Daf1-/-C3-/- mice. Importantly, the increased T cell activity upon antigen re-stimulation *in vitro* was lost in Daf1-/- mice after neutralisation of C5 using C5-blocking antibodies *in vivo*. This finding implies that complement products downstream of C5, i.e. the anaphylatoxin C5a or the MAC, are responsible for the observed phenotype in the Daf1-/- mice.

Heeger *et al.* independently reported similar findings in a different line of Daf1-/- mice (Heeger *et al.*, 2005). In this study, the authors observed that Daf1-/- spleen cells proliferated more vigorously following *in vitro* stimulation with allogeneic cells and more spleen cells purified from female mice immunised with the male antigen (HY*Dby*) produced IFNγ upon restimulation *in vitro*. In addition, Daf1-/- female mice rejected male skin grafts more efficiently than their WT counterparts and exhibited more HY-specific T cell activity as measured in IFNγ ELISpot assays.

Liu *et al.* showed that the effects of Daf1 deficiency on T cell activity are lost when complement activity is removed, either by back-crossing to C3-/- mice or by

inhibition of C5 (Liu *et al.*, 2005). Heeger *et al* reported similar findings, though in these experiments, neutralisation of C5 only partially abrogated the effect of Daf1-deficiency (Heeger *et al.*, 2005). They also showed that T cell responses were partially normalized in Daf1-/-Factor D-/- mice, thereby indicating that the alternative pathway of complement activation contributes to the influence of Daf1 on T cell activity.

Overall, complement activation plays a critical role in T cell modulation by Daf1. Complement components not only mediate immune defence against foreign pathogens, but also modulate diverse processes, such as cell survival, growth, and differentiation in various tissues (Mastellos and Lambris, 2002). Complement has been shown to be critical for liver regeneration after acute toxic challenge. Local production of C3a and C5a induce, through interaction with their receptors, downstream activation of the latent transcription factors NF-κB and STAT-3 necessary for hepatocyte regeneration (Markiewski *et al.*, 2004; Strey *et al.*, 2003). In addition, synthesis of factor D, B and C3 by adipose cells and consequent activation of the alternative pathway of complement, has been related with adipose cell metabolism (Choy *et al.*, 1992). In the absence of Daf1, exacerbated complement activation result in generation of several proteolytic fragments with biological activity such as C3a, C3b and C5a (Figure 1.3). Indeed, *in vitro* activation of T cells induce the release of complement components of the alternative pathway C3, factor B and D (Heeger *et al.*, 2005). The direct mechanism with which complement drives T cell activation remains unclear. Neutralization of C5 abrogates Daf1-/- phenotype suggesting a role for C5a and/or MAC. C5a, a potent anaphylatoxin, is known to promote and perpetuate inflammatory reactions by facilitating chemotaxis of neutrophils, eosinophils, monocytes, macrophages and lymphocytes and by inducing mast cells, neutrophils and

macrophages to release inflammatory cytokines such as IL-12, TNFα and IL-6

(reviewed in Guo and Ward, 2005). C5a may therefore indirectly influence T cell

function through promotion of inflammation and creating a favourable environment for

the activation of antigen-specific T cells. Although expression of C5aR on murine T

cells has not yet been described, virus-specific T cell responses are impaired in mice

treated with a peptide antagonist of C5aR (Kim *et al.*, 2004a). Neutralization of C5

also disrupts the formation of the MAC. MAC has also been implicated in T cell

activation on human T cell lines (Murray and Robbins, 1998). Neutralisation of C5 did

not completely abrogate the effect of Daf1-deficiency in some of the experiments

described above, and it is possible that C3a and/or C3b might play a role. C3a

molecule, another anaphylatoxin, has a wide spectrum of pro-inflammatory effects and

C3aR is expressed by activated T cells (reviewed in Muller-Eberhard, 1988; Werfel *et*

al., 2000). Kerekes *et al.* showed that deposition of C3b on APCs facilitates T cell

proliferation through improving cell-to-cell contact between APC and cognate T cell

(Kerekes *et al.*, 1998). Although T cells express a variety of C3 receptors, including

the C3b receptor, CR1, a systematic study of the effects of complement fragments on T

cell activity has not yet been performed (reviewed in Wagner and Hansch, 2006). Also

worthy of consideration is the possibility that a complement-independent component

may contribute to the hyperactivity of Daf1-/- T cells since C3-deficient Daf1-/- T

cells displayed enhanced responses to recall antigens compared to those purified from

C3-/- mice (Liu *et al.*, 2005). It is possible that CD55 binds to an as yet unidentified

ligand on APCs, which directly or indirectly, downmodulates T cell activation.
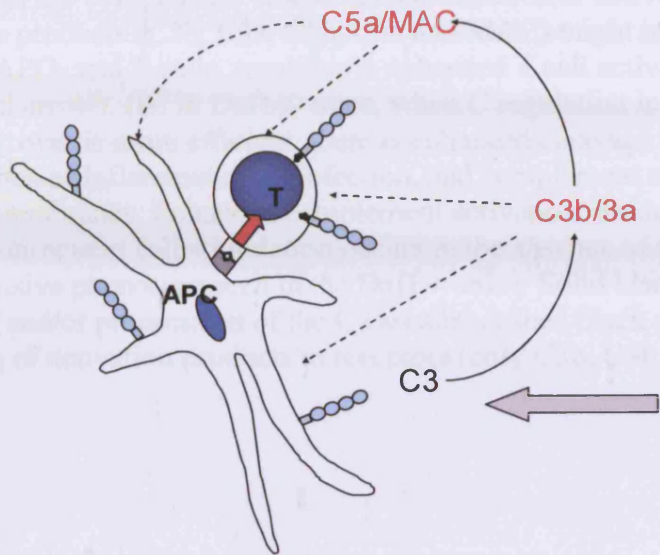
A

C5a/MAC

T

C3b/3a

APC

C3

Systemic C
(from plasma)
+
Local biosynthesis
(T cells and APCs)

B

C5a/MAC

T

C3b/3a

APC

C3

Systemic C
(from plasma)
+
Local biosynthesis
(T cells and APCs)

C5a/MAC

C3b

C3b

C3 → C3b

C5a/MAC

C3b

CReg (CD55)

TCR

MHC + peptide
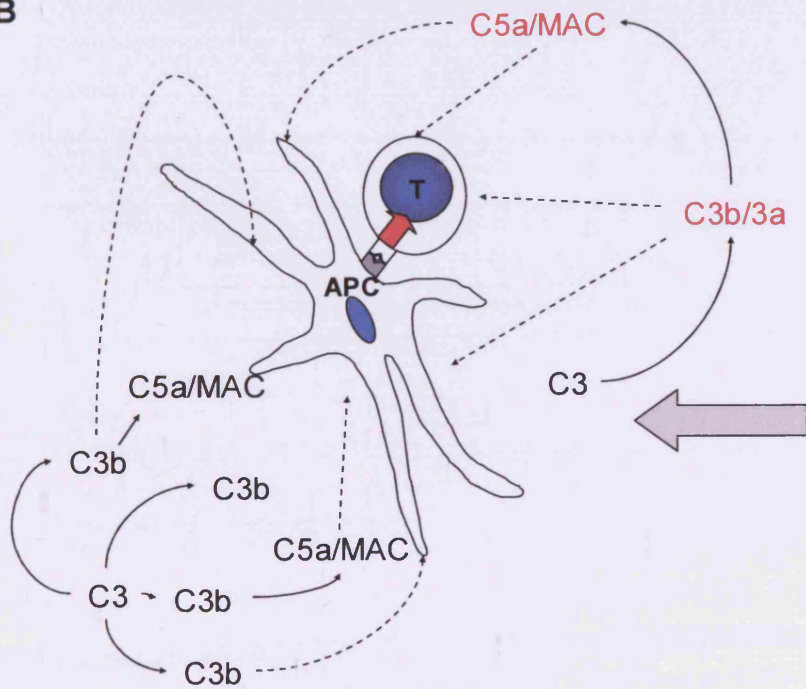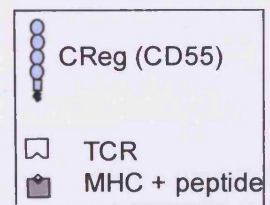
Figure 1.3. Complement Activation in Daf1-/- Mice

(A) Complement ticks over spontaneously in plasma resulting in low level production of C3b. In WT animals, in which expression of CRegs is normal, levels of active complement are low and tick-over amplification is kept in check. In inflammatory sites, activation of the complement cascade and production of active mediators is increased. These products (C3b, C3a, C5b, C5a and MAC) might have stimulatory effects on both APCs and T cells, resulting in enhanced T cell activation and proliferation (red arrow). (B) In Daf1-/- mice, when C regulation is deficient, complement tick over is more efficient, there is enhanced cleavage of C3 to C3b, particularly at sites of inflammation or infection, and complement amplifies more readily on cell membranes. Enhanced complement activation, production of active mediators and subsequent cell stimulation occurs in the absence of CD55 resulting in the hyper-responsive phenotype seen in the Daf1-/- mice. Solid black arrows indicate cleavage events and/or progression of the C cascade; dashed black arrows indicate putative binding of activation products to receptors (only C3b, C3b, C5a and MAC are shown here).

One known non-complement ligand for CD55 is CD97, a transmembrane

protein and member of the EGF (Epidermal Growth Factor)-TM7 family with an

extended extracellular region containing a variable number of EGF-like domains. It is

constitutively expressed on granulocytes, monocytes and DCs and is rapidly

upregulated in lymphocytes upon activation. CD55 binds CD97 with low affinity and a

rapid off rate. The physiological significance is still unknown but it has been suggested

that the interaction facilitates cell adhesion. Ex-vivo incubation with mAbs to CD97

delayed neutrophil recruitment to the colon in an experimental model of colitis

(Leemans *et al.*, 2004). However, Heeger *et al.* found no effect of antibody blocking of

CD97 on modulation of T cell responses (Heeger *et al.*, 2005).

1.4. GPI-anchored Proteins and T Cell Activation

1.4.1. Roles of GPI-anchored Proteins in T Cell Activation *In Vitro*

As discussed above, the way in which a GPI-anchored protein such as CD55

influences T cell activation is unclear. Although the studies described above strongly

support a role for complement components, there is also evidence that CD55 can

modulate T cell activation in a complement-independent fashion. It is interesting that

GPI-anchored proteins other than CReg have been shown to modulate T cell activity in

man and rodents implying that the effect of CReg on T cell activation is not

inextricably linked to the complement system (Gonzalez-Cabrero *et al.*, 1999; Gunter

et al., 1984; Kroczek *et al.*, 1986; Malek *et al.*, 1986; Reiser, 1990; Thompson *et al.*,

1989).

Thy-1 was the first GPI-anchored protein described to induce T cell activation (Gunter *et al.*, 1984; Kroczek *et al.*, 1986). Thy-1 exists in two allotypic forms, Thy-1.1 and Thy-1.2, and is expressed by thymocytes, T cells and brain tissue in mice. Thy-1 specific antibodies have been shown to stimulate T cell activation, measured by proliferation, IL-2 production, IL-2R expression and Ca^{2+} mobilization, directly or indirectly after cross-linking Thy1-specific antibodies in the presence of PMA (Kroczek *et al.*, 1986). Similar observations have been made with other GPI-anchored proteins. These include Ly-6 (Malek *et al.*, 1986), Qa-2 (Hahn and Soloski, 1989; Robinson *et al.*, 1989), CD48 (Maschek *et al.*, 1993; Reiser, 1990), CD73 (Massaia *et al.*, 1990; Thompson *et al.*, 1989) and as described above, CD55 and CD59 (Korty *et al.*, 1991; Shenoy-Scaria, 1992). Ly-6 comprises a family of cell surface proteins of unknown function found in mouse lymphocytes. Studies show that antibody cross-linking of Ly6 with Fc receptors expressed on APCs or secondary antibodies, results in IL-2 production, IL-2R expression and T cell proliferation (Malek *et al.*, 1986). Qa-2 is a non-classical mouse MHC class I molecule expressed at high levels on T cells. Unlike the classical class I molecules, H-2 K, D and L, Qa-2 lacks a transmembrane domain and is attached to the cell membrane by a GPI-anchor. Antibody cross-linking of the Qa-2 antigen but not H-2 antigens induced T cell proliferation in the presence of PMA (Hahn and Soloski, 1989; Robinson *et al.*, 1989). The GPI-anchored protein CD48 (formerly known as sgp-60) is expressed on lymphocytes, macrophages and DCs. Antibody cross-linking of CD48 results in T cell proliferation, IL-2 production and Ca^{2+} mobilisation following stimulation with CD3 specific antibodies and PMA (Maschek *et al.*, 1993; Reiser, 1990).

1.4.2. Roles of GPI Anchored Proteins in T Cell Activation *In Vivo*

The role of GPI-anchored proteins in T cell activation is illustrated in mice deficient in specific GPI-anchored proteins. In the case of Thy-1- and CD48- deficient mice, T cells, stimulated with allogeneic cells or CD3-specific antibodies tended to proliferate less well than their WT counterparts. Reduced proliferation was accompanied by reduced tyrosine phosphorylation and Ca^{2+} influx compared to T cells from WT mice (Beissert et al., 1998; Gonzalez-Cabrero et al., 1999). Different results were obtained in a similar study using Ly6A-deficient mice (Stanford et al., 1997). Splenocytes from the knockout mice exhibited enhanced T cell proliferation compared to WT mice after stimulation with allogeneic splenocytes, CD3-specific antibodies, KLH or ConA. The ability to generate allogeneic CTL responses was also tested with no significant distinction observed between the different groups of mice. In support of the premise that GPI-anchored proteins modulate T cell activity is the observation that T cell proliferation is enhanced in lymphocytes lacking GPI-anchored proteins (Hazenbos et al., 2004). The effects of different GPI-anchored proteins on T cell activation are summarised in Table 1.1.

1.4.3. Modulation of T Cell Responses – A General Role for GPI Anchored Proteins?

Overall there is a general consensus that GPI-anchored proteins modulate T cell activation but the specific influence of each of the different proteins remains controversial. In general, cross-linked antibodies to GPI-anchored proteins induce T cell activation but studies of T cells from mice lacking certain GPI-anchored proteins have revealed that the proteins may positively (Thy-1 and CD48) or negatively (CD55 and Ly6A) modulate T cell activation in vivo. Despite the lack of a cytoplasmic signalling domain, these proteins are, as described above, believed to facilitate

GPI Protein	Target	Stimulation	Effect	Reference
Thy1.1	Mouse T cells	Antibody cross-linking + PMA	Proliferation and Ca++ influx	Kroczek *et al.*, 1986
	Mouse T cells (Thy1.1 -/- mice)	Allogeneic cells or mAbs to CD3	Thy1.1 -/- T cells exhibit decreased proliferation, Ca++ influx and Tyr phosphorylation	Beissert *et al.*, 1998
Ly6	Mouse T cells	Antibody cross-linking + PMA or mAb to CD3	Proliferation, IL-2 release and IL2-R expression Ca++ influx	Malek *et al.*, 1986 Stanford *et al.*, 1997
	Mouse CD4$^+$T cells (Ly6 -/- mice)	Allogeneic cells or mAbs to CD3 or ConA or KLH antigen	Ly6-/- T cells exhibit increased proliferation	Stanford *et al.*, 1997
Qa-2	mouse T cells	Antibody cross-linking + PMA	Proliferation	Hahn and Soloski, 1989; Robinson *et al.*, 1989
CD48	Mouse CD4 $^+$ T cells	Antibody cross-linking + PMA	Proliferation	Maschek *et al*, *1993*
	Mouse T cells	Fab to CD48+ mAb to CD3 + PMA or ConA	Inhibition of cell proliferation, IL-2 release, Ca++ flux	Reiser, 1990
	Mouse CD4$^+$T cells (CD48-/- mice)	Allogeneic cells, PHA, ConA or mAbs to CD3	CD48 -/- T cells exhibit decreased proliferation	Reiser, 1990
CD73	Human T cells	Antibody cross-linking + PMA	Proliferation, IL-2 release and IL2-R expression	Thompson *et al.*, 1989

Table 1.1. Effect of GPI-anchored Proteins in T Cell Activation

Data was obtained either from antibody cross-linking experiments or using T cells purified from genetically modified mice lacking individual GPI-anchored proteins.

intracellular signalling through association with tyrosine kinases in lipid rafts. The diverse effects of different GPI-anchored proteins may reflect the composition of proteins and signalling molecules present in the raft that they occupy. Thus, the presence of distinct groups of proteins within rafts allows specific molecules to interact in response to a given stimulus and influence the biological outcome.

1.5. Studies of CD59 *In Vivo*

Ligation of CD59 on human lymphocytes induced Ca^{2+} flux, IL-2 release and cell proliferation suggesting a role of positive modulation of T cell activation. This effect was attributed to the recruitment of signalling molecules in particular Src kinase family members. However, as discussed previously for other GPI-anchored proteins, interaction of these proteins with their natural ligand under physiological circumstances does not always correlate with *in vitro* antibody cross-linking findings. Indeed results of the detailed studies of murine CD55 using Daf1-/- mice did not support those previously obtained *in vitro* using human T cells. Whereas, antibody cross-linking of CD55 promotes T cell activation, mice genetically deficient for CD55 (Daf1) exhibited enhanced T cell responses. Whether this observation reflects a species difference or the difference between antibody cross-linking and engagement with more natural ligands remains to be elucidated.

For activation, naive $CD4^+$ T cells require the engagement not only of their TCR with MHC peptide complexes (signal 1), but also a costimulatory receptor, such as CD28 with ligands such as B7.1 (signal 2) (Dubey *et al.*, 1996; Harding *et al.*, 1992; Horgan *et al.*, 1990). In addition, several other interactions between the T cell and the

APC are known to influence T cell activation either positively or negatively, presumably altering the threshold of signals required for activation to occur (reviewed in Kroczek *et al.*, 2004; Leibson, 2004)). It appears that GPI-anchored proteins, including CReg, can perform this function. To further our understanding of the effect of CReg on modulation of T cell activity *in vivo*, this thesis describes a study of the effect of the murine CReg, CD59a on T cell activity. To achieve this goal, mice lacking CD59a were studied. Antigen-specific T cell activity was measured following infection of mice with recombinant vaccinia virus (Model 1) and infection with influenza virus (Model 2). Use of these models, described in detail below, allow the effect of CD59a on both antigen-specific $CD4^+$ and $CD8^+$ T cells to be studied. In addition, the models allow an examination of whether modulation of T cell activity through expression of CReg alters the course and/or pathogenesis of virus infection.

1.6. Model 1: Vaccinia Virus

1.6.1. The Virus

Vaccinia virus (VV) is a member of the poxvirus family, a complex family of viruses containing a large double-stranded DNA genomes. VV contains approximately 200 genes which can be subdivided into three temporally distinct classes termed early, intermediate, and late (Johnson *et al.*, 1993; Moss, 1990). Early genes are transcribed immediately after infection by enzymes and transcription factors contained within the infecting virion, while late and intermediate genes are transcribed only after the start of viral DNA replication (Moss, 1990). The genes located in the central region of the genome are highly conserved and encode enzymes, transcriptional factors and

structural proteins necessary for virus replication. The VV genome also contains more variable genes that are in general non-essential for virus replication but which affect the immune response of the host (Perkus *et al.*, 1991). Indeed, VV have unique strategies for evasion of the host immune response (McFadden *et al.*, 1995) reviewed in (Alcami and Smith, 1995; Spriggs, 1996) and are the only virus family known to produce secreted versions of receptors for cytokines such as TNF, IL-1β, IFN-α/β, and IFN-γ that bind cytokines with high affinity blocking their activity.

Viral particle formation is a complex process occurring in the cytoplasm of infected cells which results in the formation of two infectious forms of the virus termed intracellular mature virus (IMV) and intracellular enveloped virus (IEV). Initially, the viral genome is surrounded by crescents which extend to form spherical IMV. Some IMV particles become wrapped by membranes derived from the intermediate compartment to form an IEV(Sodeik *et al.*, 1993). VV is thought to leave the cell by fusion of the outer IEV membrane with the plasma membrane, to give rise to the extracellular enveloped virus (EEV) (Morgan, 1976).

1.6.2. Immune Response to VV

VV can cause either localized, self-limited infection or more generalized infection. VV has a broad cellular tropism and infects cells of many different mammalian species (Buller and Palumbo, 1991). It has been shown to infect multiple organs in the host such as skin, liver, lung and ovaries. It has also been shown to infect human DCs, NK cells, monocytes/macrophages, B cells and activated T cells but not resting T cells (Chahroudi *et al.*, 2005; Kirwan *et al.*, 2005). This suggests that the cell surface

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receptor for VV is ubiquitously expressed and highly conserved; however, no receptor for VV has been reported.

VV is a potent inducer of IFN and nitric oxide synthase (iNOS) production by macrophages (Harris *et al.*, 1995; Karupiah *et al.*, 1993). Also, several cytokines have been shown to play an important role in host defences against VV infection. Some cytokines contribute to increased virulence, as with IL-4, whereas others have a protective role against VV infection, as IFN-γ, TNF-α, IL-12 and IL-18 (Gherardi *et al.*, 2003; Gherardi *et al.*, 1999; Ramshaw *et al.*, 1997; Sharma *et al.*, 1996).

VV elicits strong and long-lasting $CD4^+$ and $CD8^+$ T-lymphocyte responses, as well as humoral immune responses (Amara *et al.*, 2004; Combadiere *et al.*, 2004; Demkowicz *et al.*, 1996; Eichner, 2003; Harrington *et al.*, 2002). During acute infection, $CD4^+$ T cell-dependent Ab response play an important role in virus clearance (Xu *et al.*, 2004b). VV infection of CD4- or IgM-deficient mice resulted in increased levels of virus titres compared to WT and virus was controlled by the action of $CD8^+$ T cells. The activity of $CD8^+$ T cells appears to be independent of perforin- or Fas-dependent cytotoxicity and be mediated by nonlytic T cell-dependent soluble mediators such as cytokines (IFN-γ) (Kagi *et al.*, 1995).

1.6.3. Recombinant VV

VV are commonly used for the generation of live recombinant viruses with the capacity to express multiple genes (Mackett *et al.*, 1982). VV can accept as much as 25

kb of foreign DNA, making it useful for expressing large eukaryotic and prokaryotic genes.

VV are attractive as vaccine vectors for the treatment of different diseases. Attenuated and host range-restricted VV that undergo limited replication in human and other mammalian cell lines, such as modified vaccinia virus Ankara (MVA) and canarypox, have been developed as vaccine vectors for use in humans. These poxvirus vectors are less pathogenic and have been shown to be safe for use in humans and to induce protective immunity in animal models (Belyakov *et al.*, 2003; Moss, 1996; Paoletti, 1996).

1.6.4 Usefulness of rVV for The Study of the Effect of CD59a on T Cell Activity *In Vivo*

As described above VV can be readily used as a viral vector for the expression of foreign proteins. An rVV expressing the glycoprotein (GP) of lymphocytic choriomeningitis virus (LCMV) has been described previously (Bachmann *et al.*, 1994). The usefulness of this rVV (rVVG2) is that the GP of LCMV contains well characterised peptide epitopes presented by H-2^b MHC class I and class II molecules that are presented to CD8$^+$ and CD4$^+$ T cells respectively. Since Cd59a -/- mice were generated on a B6 background, use of rVVGP allows comparison of both CD4$^+$ and CD8$^+$ T cell responses to be measured and compared to those observed in control B6 mice. In addition, the main site of rVV replication in female mice is known to be the

ovaries. Thus, use of this model also facilitates measurement of T cell responses at the site of infection and allows any effects on control of virus infection to be monitored.

1.7. Influenza A Virus

1.7.1. The Virus

Influenza virus is an enveloped RNA virus from the Orthomyxoviridiae family measuring 80 to 120 nanometers in diameter. There are three strains of influenza virus, A, B, and C, each characterized by the antigenic properties of its internal, non-glycosylated components. Influenza A and B are the strains that cause epidemic human disease whereas type C virus is associated with minor symptoms. Influenza A viruses can be further categorized into subtypes on the basis of two surface antigens present in the lipid bilayer: hemagglutinin and neuraminidase.

The core of the viral particle consists of ribonucleoproteins surrounded by a lipid bilayer that contains two types of glycoproteins; neuraminidase (NA), that facilitates release of viral progeny from infected cells, and haemagglutinin (HA), which is responsible for binding the virus to red blood cells and other host cells. It is now recognized as the crucial antigen recognised by the host's immune system. The influenza A genome consists of eight separate pieces of single-stranded RNA (McGeoch et al., 1976), a feature which increases the potential for genetic reassortment and may contribute to development of new influenza virus variants and antigenic drift (Bush et al., 1999; Ghedin et al., 2005).

The genome encodes for 10 proteins: Haemagglutinin (3 distinct

hemagglutinins, H1, H2, and H3); Neuramidase (2 different neuraminidases N1 and

N2 have been found in human viruses); Nucleoprotein (NP); two matrix proteins (M);

two non-structural proteins (NS1 and NS2) and three polymerases (PB1, PB2 and PA)

(Ghedin *et al.*, 2005).

1.7.2. Replication Cycle

Influenza virus infects the epithelial cells of the respiratory tract. Sialic acid on the

host cell interacts with the HA (Weis *et al.*, 1988) on the surface of the viral particle

that is taken into the cell by endocytosis. In the low pH environment of the endosome,

the ribonucleoprotein complex (RNP) is released into the cytoplasm, from where it is

transported into the nucleus. Influenza mRNA synthesis is initiated by the RNA

polymerase complex. Using host cell machinery, new viral proteins are translated from

transcribed mRNA in the cytoplasm. New viral RNA is encased in the capsid protein,

and together with new matrix protein is then transported to sites at the cell surface

where envelope HA and NA components have been incorporated into the cell

membrane. Within a few hours of infection, 100 or more incomplete virions can be

detected at the periphery of the cytoplasm. Progeny virions are formed and released by

budding (Figure 1.4).
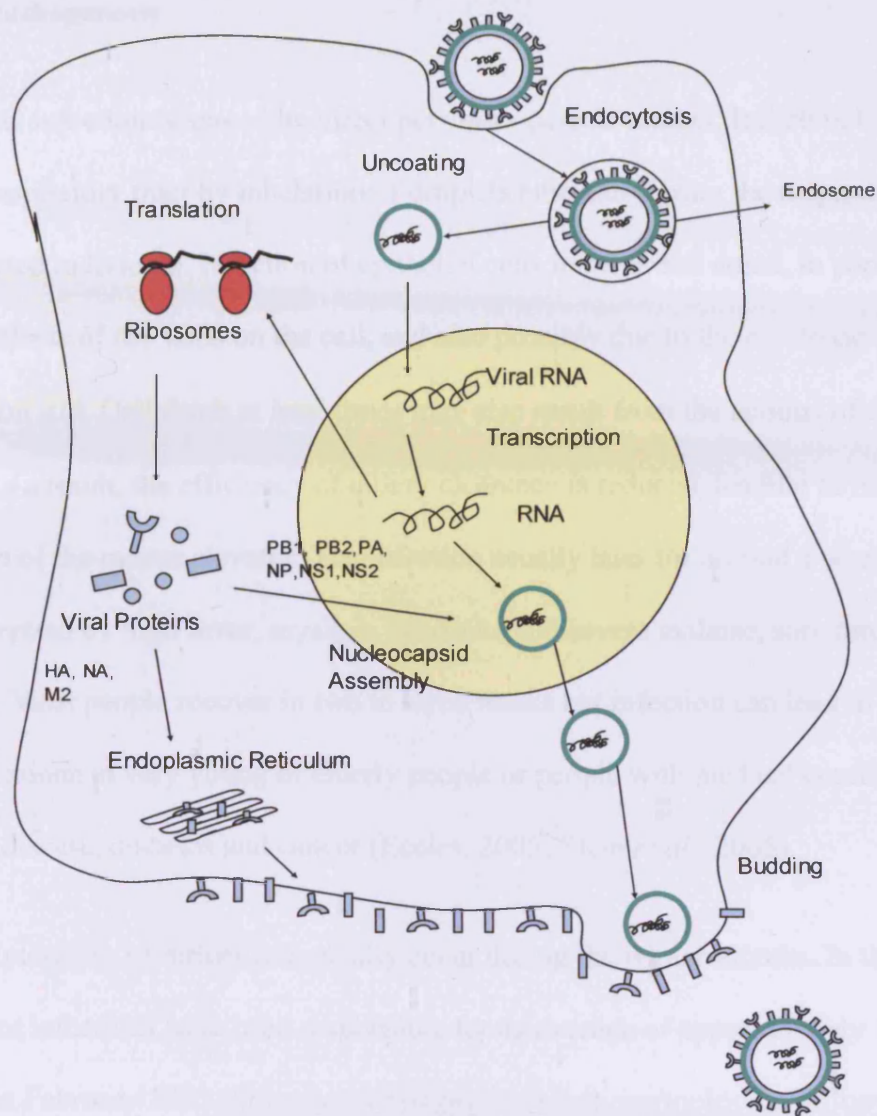
Figure 1.4. Influenza A Virus Replication
Initially, HA binds to sialic acid receptors on the host cell membrane. The virus is endocytosed, and then moved into an endosome where the low pH frees the virus nuclear material to enter the nucleus of the host cell and replicate. At the nucleus, host mRNA provides the primer for viral mRNA from which new viral proteins are synthesized. The viral mRNA is synthesized and moves into the cytoplasm for protein synthesis. Newly synthesized polymerases, NP,NS1 and NS2 are transported into the nucleus while HA, NA and M2 migrate to the cell membrane to await budding. After new viral RNA and proteins have been synthesized. The proteins and RNA segments group together in the nucleus of the host cell to form new nucleocapsids. The new nucleocapsids then migrate to the cell membrane and merge with the new matrix and glycoproteins.

1.7.3. Pathogenesis

Influenza infection is spread by direct person-to-person contact. Infection begins in the upper respiratory tract by inhalation of droplets emanating from the respiratory tract of an infected individual. Infection of epithelial cells induces cell death, in part due to the direct effects of the virus on the cell, and also possibly due to the cytotoxic effects of interferon α/β. Cell death at later times may also result from the actions of cytotoxic T cells. As a result, the efficiency of ciliary clearance is reduced, leading to impaired function of the mucus elevator. The infection usually lasts for around a week and is characterized by high fever, myalgia, headache and severe malaise, sore throat and rhinitis. Most people recover in two to three weeks but infection can lead to severe complications in very young or elderly people or people with medical conditions such as lung disease, diabetes and cancer (Eccles, 2005; Stein *et al.*, 2005).

Epidemics of influenza typically occur during the winter months. In the UK, influenza infections have been responsible for an average of approximately 13000 deaths in February 2005 (http://www.hpa.org.uk/infections/topics_az/influenza), the influenza A/Wellington/1/2004(H3N2)-like virus being the dominant circulating strain. Influenza viruses can also cause pandemics, during which rates of illness and death from influenza-related complications increase worldwide. In 1918-9 the "Spanish Flu" influenza A (H1N1) killed up to 50 million people worldwide and nearly half of those who died were young, healthy adults. The "Spanish Flu" appears to have an avian origin. In 1957 and 1968 two other influenza pandemics generated approximately 1-2 million and 700,000 death respectively and were caused by viruses containing a

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combination of genes from a human influenza virus and an avian influenza virus (http://www.cdc.gov/flu/pandemic).

1.7.4. Innate Immune Responses to Influenza Virus

The innate as well as the adaptive immune response is important for controlling influenza infection (Fujisawa *et al.*, 1987; Garcia-Sastre *et al.*, 1998). The successful generation of the innate response is also necessary for a proper transition to and development of the adaptive response.

Infected epithelial cells and macrophages in the lung respond to virus infection by deploying a complex network of signalling molecules that initiate and then amplify the production of a number of cytokines and chemokines. Experiments performed in vitro using influenza-infected human epithelial cells showed that infection resulted in the rapid induction of MIP-1α, MIP-1β, TNF-α, IL-6 and IL-8 gene expression (Adachi *et al.*, 1997; Arndt *et al.*, 2002; Tong *et al.*, 2003). Influenza A virus-infected human monocytes/macrophages secrete MIP-1α, MIP-1ß, RANTES, MCP-1, MCP-3, MIP-3α, and IP-10 (Bussfeld *et al.*, 1998; Matikainen *et al.*, 2000; Sprenger *et al.*, 1996). Secretion of these cytokines and chemokines contribute to the recruitment of neutrophils and mononuclear cells, and virus clearance. IL-8 recruits primarily neutrophils and stimulates their degranulation and adhesion (Storgaard *et al.*, 1997) whereas MIP-1α, MIP-1ß, and MCP-1 recruit primarily monocytes/macrophages (Menten *et al.*, 1999; Rollins, 1992). TNF-α exerts powerful anti-influenza virus activity. Seo and colleagues demonstrated that TNF-α has a direct anti-influenza effect by inhibiting virus replication (Seo and Webster, 2002). Influenza viruses also

efficiently induce secretion of interferons (type I and II), which represent a major

component of the innate antiviral immune response (Garcia-Sastre *et al.*, 1998). Both

types of IFN upregulate the expression of MHC class I and II molecules and activate

NK cells (Welsh *et al.*, 1991). IFN-α/β (Type I) not only have a direct anti-virus effect

but also promote the survival of lymphocytes. IFN-γ (Type II) induces macrophages

activation. The importance of these cytokines in controlling virus infection was

demonstrated in a recent study which showed that as a mechanism of escape from the

host innate response, the influenza virus protein, NS1, can attenuate the host response

mediated by IFN-α/β and TNF-α (Garcia-Sastre *et al.*, 1998; Seo *et al.*, 2002).

It has recently been suggested that NK cells are also involved in control of

influenza infection (He *et al.*, 2004; Liu *et al.*, 2004; Siren *et al.*, 2004). Infected cells

rapidly activate NK cells resulting in production of IFN-γ and the activation of

macrophages and DCs. Activation of NK cells can occur following a direct interaction

between the Nkp46 receptor on NK cells and HA on virus-infected cells (Mandelboim

et al., 2001; Siren *et al.*, 2004) and by macrophage-derived IFN-α (Siren *et al.*, 2004).

1.7.5. Adaptive Immune Responses to Influenza Virus

The early innate immune response to influenza virus is not sufficient to control and

clear the virus. The adequate clearance of the virus requires a complex interplay

between cytotoxic T cells, neutralizing antibodies and CD4$^+$ T cell help and the

importance of each arm of the adaptive immune system depends upon the strain of

influenza virus in question.

Influenza virus infects epithelial cells and monocytes/ macrophages of the airways and these infected cells are able to process and present antigen to lymphocytes. With an adequate immune response, the virus is controlled within 7-10 days mainly by cytotoxic $CD8^+$ T cells. In a mouse model using a standard laboratory strain of influenza virus, $CD8^+$ T cells have been shown to be primed, activated and expanded in the draining lymph node 3–4 days after infection (Lawrence and Braciale, 2004; Tripp *et al.*, 1995a) and subsequently migrate to the lung at day 5-7. $CD8^+$ T cells exert their effector functions at this site, producing antiviral cytokines and lysing target cells by mechanism involving perforin and Fas (Topham *et al.*, 1997). The importance of virus-specific $CD8^+$ T cells is demonstrated by the observation that β2-microglobulin-negative mice, which are devoid of MHC class I-restricted T cells, showed a delay in virus clearance (Eichelberger *et al.*, 1991). Although these mice still recover from the infection, infection with a more pathogenic strain of influenza caused death of β2-microglobulin-negative mice (Bender *et al.*, 1992).

Less is known about the role of $CD4^+$ T cells in influenza infection. During primary influenza infection, removal of $CD4^+$ T cells had no effect on virus clearance (Allan *et al.*, 1990). However, two later studies showed a diminished clonal expansion of influenza-specific $CD8^+$ CTL in CD4-deficient MHC class II-/- mice (Riberdy *et al.*, 2000; Tripp *et al.*, 1995b). The importance of $CD4^+$ T cells became more evident in the absence of B cells. In Ig-/- μMT mice, the absence of the $CD4^+$ T cell population was associated with diminished recruitment of virus-specific $CD8^+$ T cells to the lung, delayed virus clearance, and increased morbidity (Mozdzanowska *et al.*, 2000; Riberdy *et al.*, 2000).

B cells are responsible for the generation of neutralizing antibodies to external viral coat proteins (Gerhard *et al.*, 1997). Control of virus infection appears to depend on the generation of Th-dependent antibodies since B cells or $CD4^+$ T helper cells alone are unable to protect the host from influenza infection (Mozdzanowska *et al.*, 1997). In the absence of B cells, mice survive virus infection but require an intact $CD4^+$ and $CD8^+$ T cell response (Epstein *et al.*, 1995; Epstein *et al.*, 1998; Mozdzanowska *et al.*, 1997). Together, the evidence indicates that either a T helper-dependent antibody response or a $CD8^+$ cytotoxic T (Tc) cell response can independently resolve the infection but they act more efficiently in combination. The immune response to influenza virus is summarized in Figure 1.5.

1.7.6. Influenza-induced Immunopathology

Although mounting an adequate immune response is crucial for virus clearance, over-exuberant infiltration of immune cells into the lung can result in tissue damage and pathology (Moskophidis and Kioussis, 1998; Singer *et al.*, 1972). Several investigators have observed that infection of mice with influenza virus leads to a dose-dependent decrease in locomotor activity, body weight, body temperature and survival, which correlates with the extent of immune activity and involves cells of both the innate and adaptive immune system (Conn *et al.*, 1995; Enelow *et al.*, 1998; Wong *et al.*, 1997a). Indeed, treatment of influenza-infected mice with cyclophosphamide resulted in decreased mortality despite higher and more persistent virus titres in treated compared to untreated mice (Singer *et al.*, 1972). This finding was attributed to the immunosuppressive effect of cyclophosphamide which resulted in a less extensive cellular infiltration in the lungs of the infected mice.
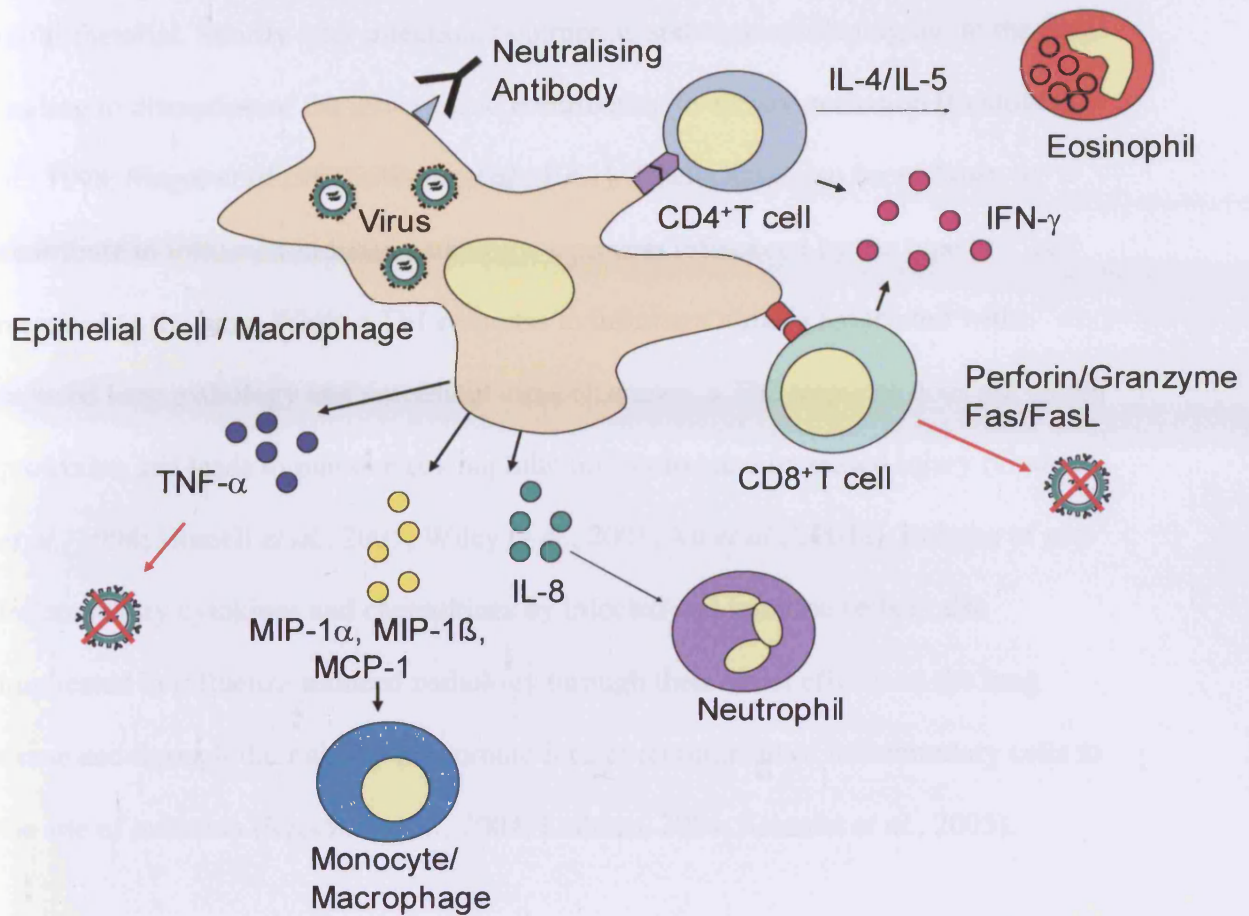
Figure 1.5. Immune Responses to Influenza Virus
Macrophages and epithelial cells release different cytokines and chemokines in response to influenza virus infection. TNF-α has a direct anti-virus effect while release of IL-8, MCP-1 and MIP-1α/β induce recruitment of monocytes, macrophages and neutrophils forming the first line of defence to influenza virus. Infected cells also present viral antigens that will be recognized by T cells initiating the adaptive response and antibodies that control virus entry to the cell.

The inflammatory sequelae associated with influenza infection appear to be multi-factorial. Shortly after infection, neutrophils and eosinophils migrate to the lungs leading to disruption of the airways and contributing to airway occlusion (Enelow *et al.*, 1998; Singer *et al.*, 1972; Wells *et al.*, 1981). T cells have also been shown to contribute to influenza-induced pathology, a process influenced by the type of T cell recruited to the lung. While a Th1 response to influenza virus is associated with reduced lung pathology and successful virus clearance, a Th2 response does not confer protection and leads to massive eosinophilic infiltration and increased injury (Graham *et al.*, 1994; Hussell *et al.*, 2001; Wiley *et al.*, 2001; Xu *et al.*, 2004a). Release of pro-inflammatory cytokines and chemokines by infected and immune cells is also implicated in influenza-induced pathology through their direct effects on the lung tissue and through their ability to promote further recruitment of inflammatory cells to the site of infection (Kroczek *et al.*, 2004; Leibson, 2004; Schmitz *et al.*, 2005).

The complement system plays an important role during the very earliest events of host defence against an invading pathogen. Indeed, complement components have been shown to contribute to virus-specific immune responses in the lung and to lung injury (Abe *et al.*, 2001; Czermak *et al.*, 1998; Kim *et al.*, 2004b; Kopf *et al.*, 2002; Krug *et al.*, 2001). C3a and C5a can act as chemoattractants of neutrophils (Martin *et al.*, 1997; reviewd in Guo and Ward, 2005) and blockade of C5a and C3a in an allergic asthma model was shown to dramatically decrease neutrophil recruitment to the airways (Baelder *et al.*, 2005; Karp *et al.*, 2001). Complement also influences the activity of influenza-specific T cells. Mice deficient in complement component C3 displayed reduced influenza-virus specific T cell responses in the lungs of infected mice, implying that components of the complement system play a role in T cell

activation and/or recruitment. Collectively, these studies imply that the complement system is likely to impinge on the extent of cellular infiltration into the lungs of influenza-infected mice and therefore on immunopathology. It is therefore reasonable to hypothesise that CReg, including CD59a, play an important role in limiting immunopathology by limiting the activation of complement. This hypothesis was tested as described in Chapter 4.

1.8. Objectives

CD59 acts as a Creg protein inhibiting the formation of the MAC and, therefore, regulating cell lysis mediated by complement. However, several studies suggest that CD59 also plays a role in T cell activation. Cross-linking of CD59 with specific antibodies on human T cells promotes T cell activation measured by Ca^{2+} flux, IL-2 production and cell proliferation. The way in which a GPI-anchored protein such as CD59a, with no cytoplasmic tail, influences T cell activation is unclear. Several studies suggest that this activation may proceed through association with src-family tyrosine kinases resulting in tyrosine phosphorylation of intracellular substrates. The physiological significance of these findings had not been investigated and it is possible that antibody cross-linking of human CD59 *in vitro* might not reflect CD59 engagement under more physiological circumstances. Thus, the first aim of this study was to investigate the effect of CD59 on T cells *in vivo*. To achieve this objective, I utilised CD59a deficient mice, which were infected with either recombinant vaccinia viruses or with influenza virus. The effect of CD59a on T cell activity *in vivo* was

therefore assessed by comparing anti-viral T cell responses in wild-type (WT) and CD59a -/- mice. These experiments are desribed in Chapters 3 and 4.

Under conditions of sustained complement activation such as tissue injury or pathogen infection, T cells are constantly exposed to potentially immunostimulatory complement fragments that affect T cell responses. It is possible therefore that as a complement regulator protein, CD59 could modulate T cell activation indirectly by modulating the extent of complement activation. With this in mind, experiments were performed to determine the extent to which modulation of T cell responses by CD59a were due to the indirect effects of CD59 on complement activation. This work, described in Chapters 3 and 4, was also carried out in the context of the virus models mentioned above. The findings of the studies described above prompted a closer analysis of the effect of CD59a on T cell activity. This work is discussed in Chapter 5.

CRegs, as explained above, have been shown to activate human T cells upon antibody ligation. A more detailed analysis of the effect of CD59 expression on the activity of human T cells was also therefore analysed. This work is described in Chapter 6.

In summary therefore, the work presented in this thesis describes an investigation of the effect of the CReg, CD59, on T cell activation following virus infection of CD59a-deficient mice. Subsequently, this study explored the mechanisms through which CD59a might affect T cell activation and finally, experiments were performed to validate the findings obtained in mice using human T cells.

Chapter 2- Materials and Methods

2.1. Mice

C57BL/6 (H-2^b) mice (WT) were obtained from Harlan (Oxford, UK). B6.129-

Cd59a^{tm1Bpm} (Cd59a-/-) mice were generated as previously described (Holt *et al.*, 2001)

and back-crossed onto the C57BL/6 background for 8 generations. Cd59a-/- mice were

intercrossed with the B6.129S4-C3^{tm1Crr} (C3-/-) mice (Wessels *et al.*, 1995) also on the

C57BL/6 background. Cd59a-/- mice on the Balb/c background were kindly provided

by Professor Marina Botto, (Imperial College, London). During experimental

procedures mice were housed in Scantainers. Experiments were performed in

compliance with Home Office regulations.

2.2. Cell Culture

2.2.1. Cell Lines

Yac-1 cells (TIB-160;American Type Culture Collection, Rockville, MD), T cells and

V8E cells, a mouse CD4^+ T cell hybridoma (Oxenius, 1997) (kindly provided by Dr

Annette Oxenius, University of Zurich, Switzerland), were maintained in RPMI

medium supplemented with 10% fetal calf serum (FCS), penicillin-streptomycin, L-

glutamine, non-essential amino-acids (Life Technologies, Grand Island, NY) and 50

μM of 2β-mercaptoethanol (2β-ME) (Sigma-Aldrich, St Louis, MO) (R10). In the

case of V8E cells transfected with CD59a, hygromycin B (Life Technologies, Grand

Island, NY) was added to the media at a final concentration of 0.3mg/ml in order to

maintain expression of CD59. TK- cells were maintained in DMEM medium supplemented with 10% FCS, penicillin-streptomycin and L-glutamine (Life Technologies, Grand Island, NY) (D10).

2.2.2. Dendritic Cells

Dendritic cells (DCs) were prepared from bone marrow cells that were collected by removing the femurs of mice, cutting off the ends of the bones, and flushing out the bone marrow with RPMI 1640 medium injected with a syringe. Cells were centrifuged at 1500 rpm for 5 min, resuspended in 12 ml of R10 and incubated 30 min at 37°C in 10 cm tissue dish. Non-adherent and loosely adherent cells were recovered, resuspended in 50 ml of medium and incubated for 6 days in medium supplemented with 10% FCS, 200 U/ml recombinant mouse Granulocyte Macrophage-Colony Stimulating Factor (rmGM-CSF; Peprotech, Rocky Hill, NJ) in a 24 multiwell plate. On day 3 of culture, floating cells were gently removed, and fresh medium was added. For DC maturation, 2μg/ml of LPS (Sigma Chemical Co., St. Louis, MO) or 50 HAU/ml of influenza virus was added to the wells at day 5. On day 6 of culture, non-adherent and loosely adherent DC cells were removed for analysis and for cell proliferation. DC purity was evaluated with CD11c staining by flow cytometry (>90%).

2.2.3. PBMCs

Peripheral blood mononuclear cells (PBMCs) from healthy donors were collected in heparin and separated by density centrifugation. 15-20 ml of blood was layered onto 20 ml of Ficoll-Hypaque solution (Pharmacia Biotech, Alameda, CA) and centrifuged at 2000 rpm for 20 min. After centrifugation, the PBMC interface was aspirated and

washed two times in RPMI 1640 medium. The PBMCs were then used for CD4$^+$ T cell

purification by positive MACS MicroBeads selection (Miltenyi Biotech, Bergisch-

Gladbach, Germany) according to the manufacturer's instructions. Cells were cultured

in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10% heat-

inactivated autologous serum, penicillin-streptomycin, L-glutamine, non-essential

amino-acids and 50 μM of 2β-ME (Sigma-Aldrich, St Louis, MO).

2.3. Cell Transfection

2.3.1. Electroporation Protocol

V8E cells were resuspended at 4x10^6 cells in 500 μl of RPMI medium without FCS.

Cells were mixed with 10 μg of plasmid containing the Cd59a gene or control vector

(pDR2Δ EF1α; gift of Dr Anegon, INSERM U437, Nantes, France) in a 0.4 cm

cuvette for 15 min on ice and then electroporated at 274v, 950 μF using the Bio-Rad

Genepulser with capacitance extender. The cuvette was then placed on ice for an

additional 5 min and cells were transferred into a sterile flask and cultured for 24 hours

in 10 ml fresh RPMI containing 10% FCS. Cells were washed once in PBS and

resuspended in selection medium (R10 and 600 μg/ml hygromycin B; Life

Technologies). After 2 weeks all of the nontransfected control cells had died.

Transfected cells were then maintained in RPMI containing 10% FCS and 300 μg/ml

hygromycin B.

2.3.2. Lipofectamine Protocol

A plasmid containing FLAG-FasL was obtained from Professor Gavin Screaton (Imperial College, London). 293T cells were transfected with the plasmid using Lipofectamine (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Cells were cultured in serum free medium supplemented with penicillin and streptomycin for 5 days at 37°C. Conditioned medium from 293T cells transiently transfected with FLAG-FasL expression vector was collected, aliquoted and used for induction of T cell apoptosis.

2.4. Induction of Apoptosis

Apoptosis was induced with FLAG-FasL (sFasL) in the presence of FLAG M2-specific antibodies to aggregate FasL. Optimal concentration of sFasL conditioned media was first tested on Jurkat cells. Splenocytes or purified $CD4^+$ T cells were treated with increasing amounts of sFasL in the presence of 5 µg/ml anti-FLAG antibody (Sigma-Aldrich, St Louis, MO). The cells were incubated for 24 hours at 37°C before assessing final cell viability using annexin/ PI staining (BD PharMingen, San Diego,CA) by flow cytometry.

2.5. Fluorescence Staining

2.5.1. Antibodies

The mAbs to CD28 (37.58) and CD3 (500A2) were purchased from Leinco Technologies. Anti-CD4Cy5, anti-CD8-Cy5, anti-F4/80-Cy5 mAbs were purchased from Caltag Laboratories (Burlingame,CA). Anti-Fas, anti-MHC-II, anti-IFNγ–FITC,

anti-CD25-PE, anti-TCR-PE, anti-CD86-FITC, anti-Gr1-PerCPCy5.5, anti-CD11c-APC, anti-CD11b-FITC, anti-NK1.1-PE, anti-B220-FITC mAbs were purchased from BD PharMingen (San Diego,CA). Donkey anti-mouse-PE and donkey anti-goat-FITC were purchased from Jackson laboratories (Bar Harbor, ME). Antibodies to human CD45RO-Cy5, CD45RA-Cy5, CCR7-PE, CCR5-FITC and CD69-FITC were purchased from BD PharMingen (San Diego,CA). Isotype controls antibodies rat IgG1, rat-IgG1-biotin, rat IgG2a-FITC were purchased from Caltag Laboratories (Burlingame,CA) and rat IgG2a-PE, rat IgG-Cy5, mouse IgG-FITC, mouse IgG2a-PE, mouse IgG2a-PECy5.5, mouse IgG1-PE, mouse IgG2b-Cy5 were purchased from BD PharMingen (San Diego,CA). The mAbs specific for mouse CD59a (mCD59.1) and human CD59 (HC1 and MEM-43) were obtained from hybridomas produced in the laboratory (Harris *et al.*, 2003). For cross-linking experiments, F(ab)'$_2$ rabbit anti-rat IgG was purchased from Serotec (Oxford, UK). Influenza-specific CD8$^+$ T cells were identified using PE-labelled MHC class I tetramer (H-2D^b) loaded with the immunodominant peptide np68 (ASNENMDAM) from the influenza nucleoprotein.

2.5.2. Extracellular Staining

Cells were incubated with 0.5µg/ml of antibody in FACS buffer (PBS + 2%FSC + 2mM EDTA) for 30 min at 4°C. In cases where directly labelled antibodies were not used, secondary stains with immunoglobulin-specific antibodies were performed. After 2 washes with FACS buffer, cells were fixed with FACS Fix buffer (PBS + 2%FSC + 2mM EDTA + 2% formalin) and analysed by flow cytometry (FACS-CALIBUR®; Beckton Dickinson, CA). For CFSE (carboxyfluorescein diacetate succinimidyl ester) staining (Molecular Probes, Eugene, OR), cells were washed with PBS and incubated

for 10 min at 37°C with 0.5 μM CFSE. The reaction was stopped with FBS and cells washed twice with PBS.

2.5.3. Intracellular Staining

Intracellular cytokine staining was performed by incubating lymphocytes for 4 hr at 37°C in the presence of ionomycin (1μg/ml), PMA (20ng/ml) and monensin (3μM) (Sigma-Aldrich, St Louis, MO). Cell surface staining was performed as described before followed by intracellular staining, performed using CytoFix/CytoPerm Kit (BD, Pharmingen, San Diego,CA) according to the manufacturer's instructions. Cells were resuspended in FACS buffer and analysed by flow cytometry (FACS-CALIBUR®; Beckton Dickinson, CA).

2.6. Cytokine Enzyme-linked Immunosorbant Assay (ELISA)

IL-2 from culture supernatants was quantified using murine IL-2 ELISA development Kit (Peprotech, Rocky Hill, NJ). 96 MW plates were coated with 100 μl of capture antibody in PBS overnight (ON) at 4°C. After 3 washes in PBS containing 0.05% Tween-20, plate were blocked with 200 μl of PBS with 10% FCS for 1 hour at RT. Samples and serial dilutions of the standard were incubated for 2 hours at RT. After 5 washes with PBS containing 0.05% Tween-20, plates were further incubated with pre-mixed biotinylated IL-2-specific antibodies and avidin-HRP. Antibody binding was detected using OPD substrate (DAKO; Hamburg, Germany) and the absorbance read at a wavelength of 480 nm in a plate reader (MRX II; Dynex Technologies, Billinghurst, U.K).

2.7. Immunoprecipitation and Western Blotting

Immunoprecipitation was performed as previously described (Baalasubramanian *et al.*, 2004). Lysates of mouse CD4$^+$ or CD8$^+$ T cells were incubated with 10 µg of mouse mAb against CD59a (mCD59.4) (Harris *et al.*, 2003) and 50 µl of protein A-Sepharose beads for 2 h at 4°C .The immunoprecipitates were washed in lysis buffer and boiled in non-reducing SDS-PAGE sample buffer. Beads were removed by centrifugation, supernatants resolved on non-reducing 12% SDS-PAGE, and transferred to a PVDF membrane (Amersham Pharmacia Biotech, Freiburg, Germany). The membrane was blocked in 5% non-fat milk in PBS for 1 hour at room temperature (RT) and probed with the CD59a-specific mAb mCD59.1, ON at 4°C. The membrane was washed three times with 0.5% Tween in PBS. The membrane was subsequently incubated with secondary antibody Donkey-anti mouse-HRP (1:10000 Jackson Laboratories, Bar Harbor, ME) for 2 hours RT, washed in 0.5% Tween PBS . Protein bands were detected with ECL Advance Western Blot detection reagent (Amersham Pharmacia Biotech, Freiburg, Germany) used according to the manufacturers instructions.

2.8. Exogenous Incorporation of GPI-CD59a

Mouse CD59a was purified from EL4 cells using an immunoaffinity column by Dr B. Sivasankar as previously described (Stefanova and Horejsi, 1991). 5×10^6 CD4$^+$ T cells were incubated with 5µg/ml of GPI-CD59a for 20 min at 37°C to permit incorporation via the GPI anchor. Cells then were washed and incubated for another 2 hours to allow incorporation into lipid rafts (van den Berg *et al.*, 1995).

2.9. CD59a Cross-linking

V8E, a mouse CD4$^+$ T cell hybridoma known to be negative for mCD59a expression by FACS, was transfected with mCD59a as described above (Harris *et al.*, 2003). Purified CD4$^+$ T cells from Cd59a-/- or WT mice or V8E cells, untransfected or transfected with CD59a, were incubated with mCD59.1 or isotype control mAb for 15 min at 4°C. After two washes in RPMI, cells were plated at 10^5 cells/well in triplicate in a 96 MW plate. Where appropriate, 5μg/ml of F(ab')$_2$ rabbit anti-rat antibody (Serotec, Oxford, UK) was added to the wells. Cells were activated with 0.5 ng/ml of PMA and incubated at 37°C. After 18 hrs, supernatant was taken and IL-2 release analysed by ELISA performed according to the manufacturer's instructions (BD, Pharmingen, San Diego,CA). For human CD59 cross-linking, purified CD4$^+$ T cells (10^5/well) were incubated with HC1 or MEM43 antibodies for 15 min at 4°C, washed and cross-linked with polyclonal rabbit serum to mouse immunoglobulins.

2.10. Complement Assay

2.10.1. Complement Inhibition

For inhibition of complement activity *in vitro*, 1 μg/ml of recombinant human soluble CR1 (sCR1; gift of T Cell Sciences) was added to each well of a 96 MW plate. Cell proliferation was assayed after 3 days by thymidine incorporation. For *in vivo* inhibition, mice were injected i.v. daily with 20mg/kg of sCR1.

2.10.2. *In Vitro* Mouse Complement Haemolytic Activity

To test complement inhibition, mice were bled every day and serum was tested for complement haemolytic activity using rabbit erythrocytes (RbE) sensitized with mouse anti-rabbit antiserum. RbE were washed twice in PBS, centrifuged at 2000 g for 10 min and resuspended with 1/200 dilution of mouse anti-rabbit serum for 30 min at RT. Red cells were washed, resuspended at 2% (v/v) in complement fixation diluent (CFD; OXOID Limited, Hampshire, UK) and aliquoted into the wells of a 96-well plate (0.1 mL/well) in triplicate. Serial dilutions of the test or control sera were incubated with the RbE (100 μl/well) at 37°C for 30 minutes. Hemolysis was measured by absorbance at 415nm ([A415 (sample)- A415 (min)]/[A415 (max) - A415 (min)]) x100. Haemolytic activity in samples was tested 24 hours post-treatment.

2.10.3. *In Vitro* Complement Lysis Assay on Human Erythrocytes

Erythrocytes (E) from healthy donors were washed two times in PBS, centrifuged at 2000 g for 10 min and resuspended at 2% (v/v) in CFD. E were sensitized with polyclonal anti-CD55 antibody (100 μl) for 30 min at RT, washed, resuspended at 2% (v/v) in CFD, aliquoted into the wells of a 96-well plate (0.1 mL/well) in triplicate. When applicable, CD59-specific antibodies were added to the wells (10 μg/ml). E were then incubated at 37°C for 30 minutes with 0.1 mL dilutions of human serum in CFD and subsequently centrifuged at 800 g for 5 min. The supernatants were transferred into a fresh flat-bottom 96-well plate and their absorbance at 415 nm was measured. Percentage of lysis for individual wells was calculated from the following formula: % lysis = ([A415 (sample-A415 (min)]/[A415 (max) - A415 (min)])x100

2.10.4. Measurement of Intravascular Haemolysis and Haemoglobinuria

Mouse blood (200 μl) was collected into tubes containing 10 μl of 0.5 M EDTA to prevent clotting. Plasma was prepared by centrifugation at 5000 rpm for 10 min at 4°C in a micro-centrifuge, carefully removed and diluted 1:10 in 0.942 M Na_2CO_3. To determined haemoglobin concentration in plasma samples, absorbance was measured at 380 nm, 415 nm and 450 nm in a spectrophotometer. The haemoglobin concentration in grams per liter was calculated from the following formula: $cHb = 1.65$ $x A_{415} - 0.93 x A_{380} - 0.73x A_{450}$. Urine haemoglobin levels from freshly collected samples were estimated by measuring the absorbance at 414 nm in a 96 multiwell plate (100 μl).

2.11. Antibody Fab Fragment Generation and Purification

Purified MEM43 and HC1 antibodies, both IgG2a subclass, were resuspended in PBS pH 7.0 and 2mM EDTA and incubated with papain (Fluka, St Louis, MO) at an enzyme:IgG ratio of 1:25 (w/w) for different times at 37°C. The Fc fragment was removed using a HiTrap protein A HP column (Amersham Pharmacia Biotech, Freiburg, Germany). The column was equilibrated in PBS pH 7.0 and the antibody sample was applied using a syringe. The column was washed with PBS until material no longer appeared in the effluent (measured by OD 280 nm). The Fc fragment was eluted in 0.1 M citric acid buffer pH 3. Fab fragment purity was analysed on a 10% SDS gel.

2.12. Preparation of Virus Stocks

2.12.1. rVV

Recombinant vaccinia virus expressing the glycoprotein (rVVG2) of lymphocytic

choriomeningitis virus (LCMV) has been previously described (Bachmann *et al.*,

1994). rVVG2 was expanded in TK- cells. A confluent monolayer of TK- cells was

infected with 10^7 pfu/ml in 5ml of VDM media (DMEM + 10% FCS + 0.05% BSA)

for 2 h at 37°C and incubated for another 48 hours in D10. The cell were subsequently

harvested and subjected to three freeze-thaw cycles. Virus titres were subsequently

measured by plaque assay.

2.12.2. Influenza Virus

Recombinant influenza A virus strain E61-13-H17 (H17; H3N2) amplified in

embryonated chicken eggs, was obtained from the National Institute for Medical

Research, London. The virus was titrated from allontoic fluid by performing a

haemagglutination assay. Chicken red blood cells collected in Alsever's solution

(Harlan Sera-Lab, Oxford, UK), were washed three times in PBS, centrifuged for 10

min at 1800 rpm and re-suspended in PBS to obtain a 1% suspension. 50 µl of virus

dilutions were added to a V 96-multiwell plate and 25 µl of 1% red blood cells were

added to each well. The plate was incubated at RT for 45 min. The last well to show

complete haemagglutination (a hazy film of red blood cells) was considered to contain

1 HA unit in 50 µl of sample.

2.13. Virus Titres

rVVGP titres were determined in the ovaries of mice at day 3 and 8 post infection.

Ovaries were homogenized and incubated into a monolayer of TK- cells as described

previously (Gallimore, 1998). TK- cells in a 24 multiwell plate were infected with 200

µl of various dilutions of the ovary homogenate for 2 hours at 37°C. The wells were

overlaid with 1ml of D10 and incubated for a further 24 hours before staining with

Giemsa. Virus titres were calculated after counting the number of plaques in one or

more of the wells.

Influenza virus titres were determined by ELISA. Lungs were harvested at day 3

and 8 post infection, homogenised, centrifuged at 2000 g and the supernatants used to

infect Madine Darby Canine Kidney (MDCK) cells in a 96 multiwell plate. After 48

hours incubation at 37°C, the supernatants were removed by aspiration and cells were

incubated with influenza-specific antibody (Serotec, Oxford, UK), followed by anti-

mouse-HRP (1:5000; Jackson Laboratories, Bar Harbor, ME) for 1 hour at RT.

Antibody binding was detected using OPD substrate (DAKO; Hamburg, Germany)

and read at 480 nm. A positive control calibration curve was constructed using

dilutions of the titrated virus stock instead of lung homogenate.

2. 14. Virus Infection and Determination of Anti-Virus Response

2.14.1. rVVG2

Mice were inoculated via intra-peritoneal (i.p.) injection with 50 µl of rVVG2 at 10^8

PFU/ml. At day 3 and 8 after infection, ovaries were harvested for virus titres and

immuno-staining and spleens were harvested for NK assays, CTL assays and CD4$^+$ T

cell proliferation assays. For measurement of memory anti-virus responses, spleens

were harvested approximately 6 weeks after infection and CTL assays and CD4$^+$ T cell proliferation assays were performed (Figure 2.1).

2.14.2. Influenza Virus

Mice were infected intra-nasally (i.n.) with 20 HAU of Influenza virus H17 in 20 μl of PBS. At day 3 and 8 after infection, perfused-lungs were harvested for virus titres, histology and immuno-staining and spleens were harvested for NK assays, CTL assays and CD4$^+$ T cell proliferation assays. For measurement of memory anti-virus responses, spleens were harvested approximately 6 weeks after infection and CTL assays and CD4$^+$ T cell proliferation assays were performed (Figure 2.2).

2.15. T Cell Proliferation

2.15.1. Polyclonal Stimulation

CD4$^+$ ór CD8$^+$ T cells from single cell suspensions of splenocytes were purified by positive MACS MicroBead selection (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. Purity of T cells was analysed by flow cytometry (> 90%). For APCs, spleen cells were panT depleted (dynabeads, Dynal, Inc., Lake Success, N.Y.) and irradiated with 2400 cGy. CD4$^+$ or CD8$^+$ T cells (2x10^4 cells) were incubated with 10^5 APCs and 1μg/ml of anti-CD3 mAb in a 96 multiwell plate. Cell proliferation was assessed by thymidine incorporation or CFSE FACS analysis at day 3. For anti-CD28 and anti-CD3 antibody stimulation, 10^5 CD4$^+$ T cells were incubated in a 96 multiwell plate with 2 μg/ml of anti-CD28 and 2 μg/ml of anti-CD3 antibodies (Leinco Technologies, St. Louis, MO).

2.15.2. Suppression Assays using CD4⁺CD25⁺ T Cells

CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cells from single cell suspensions of splenocytes were purified using a Treg purification Kit (Miltenyi, Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's instructions. Purified CD4$^+$CD25$^-$ T cells (2×10^4 cells) were incubated with 10^5 irradiated APCs and 1µg/ml of anti-CD3 mAb in a 96 multiwell plate and CD4$^+$CD25$^+$ T cells were added to the wells in 1:1 or 1:5 ratios when applicable. Cell proliferation was assessed by thymidine incorporation at day 3.

2.15.3. Adoptive Transfer of Fluorescently Labelled Cells

Purified CD4$^+$ T cells from naïve Cd59a-/- or WT mice, were then labelled with fluorescent dyes, either PKH26 (red) or PKH67 (green) respectively (Sigma-Aldrich, St Louis, MO) according to the manufacturer's instructions. The cells were transferred to Rag-/- recipient mice by i.v. injection. One day later, the mice were challenged i.n. with 20 HAU of influenza virus. After an additional 3 days, the mice were sacrificed, and their lungs were perfused and infiltrated cells analyzed by flow cytometry.

2.15.4. Virus-specific T Cell Proliferation

Vaccinia specific CD4$^+$ T cell proliferation assays were performed by incubating 10^5 CD4$^+$T cells with 6×10^5 irradiated splenocytes and 2.5 µg/ml of P13 (GLNGPDIYKGVYQFKSVEFD) (LCMV-GP, I-A^b) or P61 (SGEGWPYIACRTSVVGRAWE) (LCMV-NP, I-A^b) peptide (EMC Microcollections, Tuebingen, Germany). For Influenza virus-specific CD4$^+$ T cell proliferation splenocytes were incubated with UV-inactivated virus. UV inactivation

Day 0   5x10⁶ PFU   **Day 0**
rVVG2 (i.p)

Cd59a-/-   WT

Day 3   NK   **Day 3**
Ovaries Inflammation
Virus Titres

Day 8   CTL   **Day 8**
CD4⁺T cell proliferation
Ovaries Inflammation
Virus Titres

Day 42   CTL   **Day 42**
CD4⁺T cell proliferation
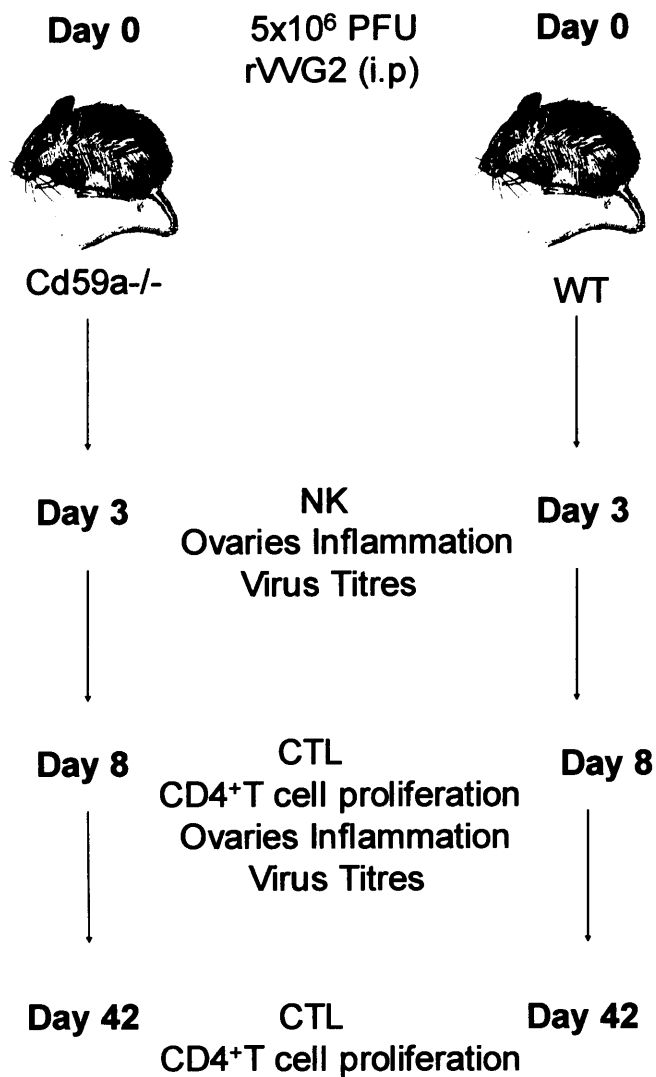
Spleen: -CD8⁺ Cytotoxic Asssay (CTL)
-CD4⁺ Proliferation Assay
-NK Cytotoxic Assay

Ovaries: -Surface/ Intracellular Staining (FACS)
-Virus Titres

Figure 2.1. Experimental Protocol. rVVG2

Figure 2.2. Experimental Protocol. Influenza Virus

was carried out by incubating the virus for 20 min under a LF-106.5 UV lamp (UVltec

Limited, Cambridge, UK). Influenza virus-pulsed DCs were also used as

APCs.Immature DCs were incubated with influenza virus (50HAU/ml) and, in some

conditions, with purified CD4$^+$ T cells and incubated for 24 hours to allow maturation.

Cells were incubated for 6 days and thymidine was added for the last 18 hours.

2.16. Killing Assay

2.16.1. CTL Assay

Spleen cells (4×10^6 cells) were stimulated *in vitro* with 1×10^6 gp33 (KAVYNFATM,

(EMC Microcollections, Tuebingen, Germany) (LCMV-GP, D^b) peptide-loaded (10^{-5}

M), irradiated splenocytes in a 24 multiwell plate and IL-2 was added at day 2. After

one week, cells were re-stimulated with 1×10^6 peptide-pulsed irrradiated splenocytes

and IL-2 10 U/ml. Five days after restimulation, cells from 6 wells were collected,

centrifuged at 1500 rpm and three-fold dilutions of the cell suspensions were made in

RPMI medium containing 10% FCS in 96 MW plate. Cells were incubated with ^{51}Cr-

labelled, gp33 peptide-loaded B16 cells (10^{-4} cells/well) for 4 h at 37°C. For minimal

and maximal lysis, splenocytes were incubated with medium or lysed with Triton

X100 respectively. rVVG2-specific CTL activity was measured by ^{51}Cr release with

the formula %lysis = ([counts sample −counts min]/[counts max − counts min])x100.

2.16.2. NK Assay

Three-fold dilutions of Spleen cells (1×10^6) were incubated with ^{51}Cr labelled Yac-1

cells (1×10^4) in 96 multiwell plates for 5 h at 37°C. For minimal and maximal lysis,

splenocytes were incubated with medium or lysed with Triton X100 respectively.

Lysis was measured by ^{51}Cr release using the formula %lysis = ([counts sample –

counts min]/[counts max – counts min])x100

2.17. Histology

Lungs were perfused with PBS and fixed in Zinc fixative (0.1M TrisHCl (pH 7.4) with

0.05% Ca acetate, 0.5% Zn acetate and 0.5% Zn chloride) (Beckstead, JH 1994). The

lungs were then embedded in paraffin wax and 5 µM sections were stained with

Hematoxylin and Eosin (H&E). Briefly, cells were stained with hematoxylin (deep

blue cells nuclei) and counter-stained with eosin (pink cytoplasm).

2.18. Statistic

All experiments were performed at least twice. Statistical significance ($p<0.05$) was

evaluated using the student t test, 2 tailed assumed unequal variance, 95% confidence

interval with the GraphPad Prism softward program. The Mann-Whitney U test was

used to determine statistical differences in lungs-histological score between groups.

Chapter 3- Murine CD59a Modulates Antiviral CD4$^+$ T Cell Activity in a Complement-independent Manner

3. 1. Introduction

Several groups have suggested that CD59 acts as a costimulatory molecule for T cell activation. Monoclonal antibody-mediated cross-linking of CD59 on PMA-treated human T cells resulted in enhanced proliferation, CD25 expression and IL-2 production (Deckert *et al.*, 1995; Korty *et al.*, 1991). Other GPI-anchored proteins have also been implicated in modulation of T cell activation (Hahn and Soloski, 1989; Kroczek *et al.*, 1986; Malek *et al.*, 1986; Marmor *et al.*, 1999 ; Maschek *et al.*, 1993; Reiser, 1990; Robinson *et al.*, 1989; Stefanova and Horejsi, 1991; Thompson *et al.*, 1989). However, studies performed *in vivo* using different GPI-anchored protein-deficient mice showed contradictory results (Gonzalez-Cabrero *et al.*, 1999; Hazenbos *et al.*, 2004; Heeger *et al.*, 2005; Liu *et al.*, 2005; Stanford *et al.*, 1997). This raised the question whether antibody cross-linking of CD59 on T cells mimics binding of CD59 to its natural ligand and whether CD59 acts as a costimulator of T cell activation *in vivo*.

In order to explore the role of CD59 in T cell activation *in vivo*, we examined T cell function in mice deficient in the species analogue, CD59a (Holt *et al.*, 2001). Cd59a-/- mice are healthy, fertile with a mild phenotype. Although mice are not anaemic, low levels of haemoglobulin can be detected in serum and urine indicating some spontaneous intravascular haemolysis. It has also been described that Cd59a-/-

mice develop, in experimental models, more severe arthritis, ischemia-reperfusion injury, allergic encephalomyelitis and nephrotoxic nephritis than Cd59a +/+ counterparts (Mead *et al.*, 2004; Turnberg *et al.*, 2004; Turnberg *et al.*, 2003; Williams *et al.*, 2004). In this chapter, T cell activity was compared in CD59a-deficient (Cd59a-/-) and WT mice following infection of mice with recombinant vaccinia virus (rVV).

For this study, we used a rVV expressing the glycoprotein (GP) of lymphocytic choriomeningitis virus (rVVG2). rVVG2 contains several MHC-restricted peptide epitopes recognized by T cells in WT mice. One peptide, gp33, derived from the GP, is presented by the MHC class I molecule, $H-2-D^b$ and is recognized by $CD8^+$ T cells (Whitton *et al.*, 1988), while another epitope, p13, is presented by the MHC class II molecule $H2-I-A^b$ to $CD4^+$ T cells (Oxenius *et al.*, 1999). Infection of mice with rVVG2 therefore allowed analyses of both $CD4^+$ and $CD8^+$ virus-specific T cell response. In this Chapter, T cell responses to rVVG2 were measured and compared in WT and Cd59 -/- mice.

3.2. Results

3.2.1 Cross-linking using Anti-CD59 mAb

Previous studies indicate that human CD59 acts as an accessory molecule for T cell

activation (Deckert *et al.*, 1995; Korty *et al.*, 1991). Cross-linking of CD59 enhanced

proliferation and IL-2 production on the Jurkat T cell line treated with PMA. In order

to confirm this finding in the mouse system, purified splenic $CD4^+$ T cells were

stimulated with PMA, CD59a-specific mAb and cross-linking anti-Ig. CD59a cross-

linking on mouse $CD4^+$ T cell did not induce IL-2 production (Figure 3.1A). Since this

may be due to the low level of CD59a on mouse lymphocytes (as assessed by FACS

(Harris *et al.*, 2003) CD59a was overexpressed in a murine $CD4^+$ T cell hybridoma

(V8E) (Oxenius *et al.*, 1999). Transfected cells expressed CD59a (Figure 3.1C) and,

when stimulated with PMA, cross-linking of CD59a yielded enhanced IL2 production

in comparison to untransfected cells subjected to the same stimuli (Figure 3.1B). These

data indicate that when CD59a is expressed at a sufficient level on murine T cells,

cross-linking CD59a does enhance IL2 production in a manner similar to that

described for human T cells.

3.2.2. Mouse T Cells Express CD59a

The laboratory has previously shown that CD59a is broadly distributed while the

second form of CD59 in the mouse, CD59b, is expressed only in testis (Harris *et al.*,

2003). In these studies it was reported that lymphocytes did not express CD59a as

assessed by flow cytometry. More sensitive methods were therefore employed to
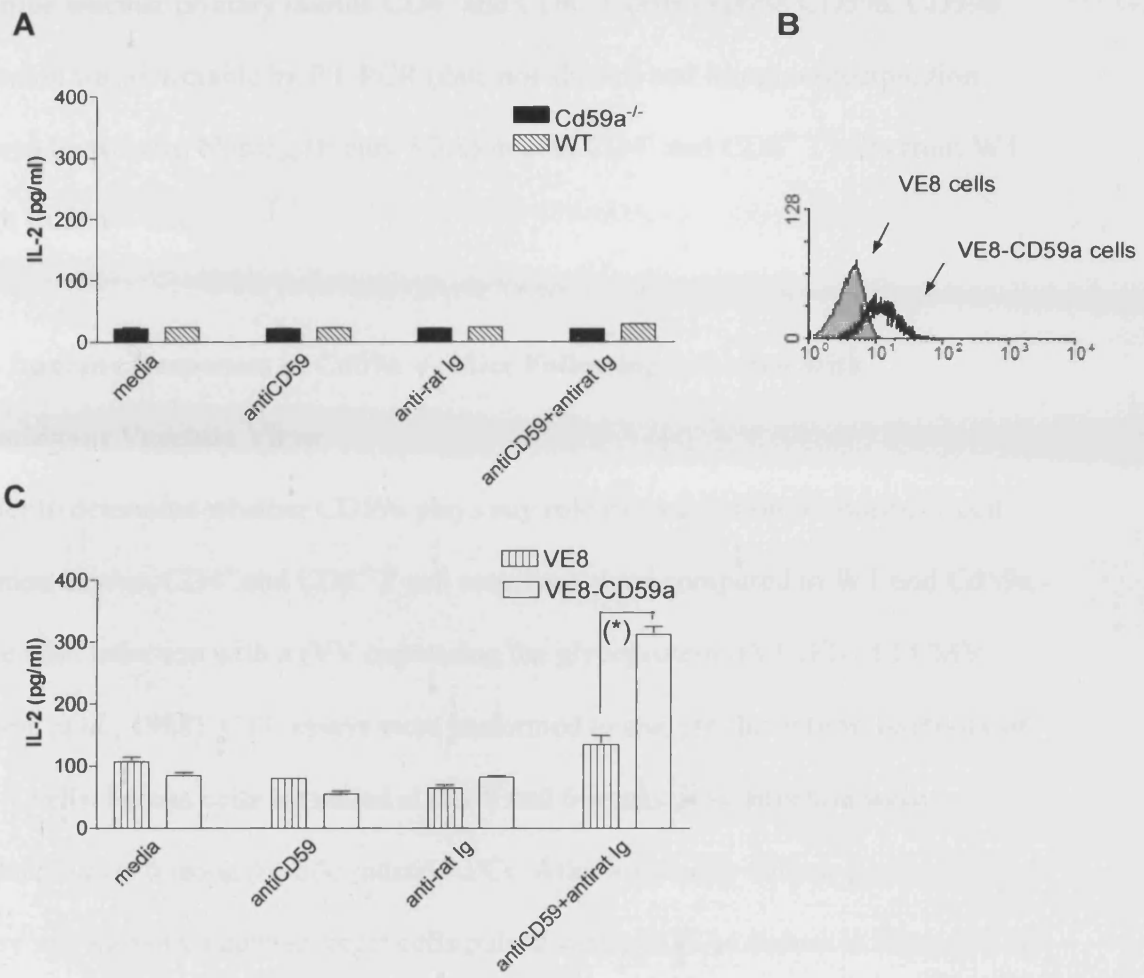
Figure 3.1. CD59a Cross-linking Induces Secretion of IL-2
Expression of CD59a inVE8 and VE8-CD59a cells was detected by flow cytometry
(B). Purified CD4^{+} splenic T cells from WT and Cd59a-/- mice (A) or VE8 and VE8-
CD59a cells (C) were incubated with mCD59.1 mAb followed by an anti-Ig Ab. After
18 hrs, IL-2 production was detected by ELISA. Values shown are mean ± SD. The
results are representative of two experiments. Statistical significance (*) was evaluated
using Student's T test (p<0.001).

determine whether primary murine CD4$^+$ and CD8$^+$ T cells express CD59a. CD59a

expression was detectable by RT-PCR (data not shown) and immunoprecipitation

followed by western blotting (Figure 3.2A) in both CD4$^+$ and CD8$^+$ T cells from WT

but not Cd59a-/- mice.

3.2.3. Immune Responses in Cd59a -/- Mice Following Infection with Recombinant Vaccinia Virus

In order to determine whether CD59a plays any role in modulation of murine T cell

activation *in vivo*, CD4$^+$ and CD8$^+$ T cell responses were compared in WT and Cd59a -

/- mice after infection with a rVV expressing the glycoprotein (rVVG2) of LCMV

(Whitton *et al.*, 1988). CTL assays were performed to analyse the antiviral-activity of

CD8$^+$ T cells. Spleen cells harvested at day 8 and 6 weeks post-infection were

stimulated *in vitro* using peptide-pulsed APCs. After a five-day culture period, CTL

activity was measured against target cells pulsed with gp33. As shown in Figures 3.2B

and D, no significant difference in CTL activity was observed between groups of mice

at either of the time-points tested. CD8$^+$ T cells also exhibited similar gp33-specific

proliferative responses as assessed by thymidine incorporation at day 8 post-infection

(Figure 3.2C). Virus-specific CD4$^+$ T cell proliferation assays were also performed at

the same time-points. As shown in Figures 2E and F, stronger proliferative responses

to the specific peptide, p13, were observed in Cd59a -/- mice compared to WT mice

whilst no differences were observed in background proliferation to an irrelevant

peptide p61, derived from the nucleoprotein of LCMV (Oxenius *et al.*, 1999).

3.2.4. Cd59a-/- Mice have Unaltered NK Cytotoxicity

A

WT   Control
CD4 CD8 Cd59a⁻/⁻   KDa

CD59a

-16.8

B

CD8

day 8

75

50

% Specific lysis

25

0

1   3   9   27

Dilution of culture

◆ Cd59a⁻/⁻
□ WT
● Cd59a-/- no peptide
○ WT no peptide

C

CD8

day 8

■ Cd59a⁻/⁻
▨ WT

10000

7500

³H-Thymidine (cpm)

5000

2500

0

media   gp33

p=0.77

D

CD8

day 42

75

50

% Specific lysis

25

0

1   3   9   27

Dilution of culture

◆ Cd59a⁻/⁻
□ WT
● Cd59a-/- no peptide
○ WT no peptide

E

CD4

day 8

■ Cd59a⁻/⁻
▨ WT

40000

30000

³H-Thymidine (cpm)

20000

10000

0

p 13   p 61

p=0.02

F

CD4

day 42

■ Cd59a⁻/⁻
▨ WT

7500

5000

³H-Thymidine (cpm)

2500

0

p 13   p 13   p 61   p 61

p=0.003

Figure 3.2. rVVGP Specific CD8⁺ T Cell Activity and CD4⁺T Cell Proliferation
CD59a expression was analyzed from immunoprecipitates of purified CD4⁺ T and CD8⁺ T cells from WT and control T cells from Cd59a-/- mice. Expression was detected using specific antibodies in a western blot (A). CD8⁺ T cell cytotoxic activity (B) and CD8⁺ T cell proliferation (C) was analyzed at day 8 after rVVGP infection. Memory CD8⁺ T cell cytotoxic activity was detected at day 42 after rVVGP infection (D). CD4⁺ T cells purified from rVVGP infected mice were tested for proliferation against peptides p61 and p13, 8 (E) and 42 (F) days post-infection. Cell proliferation was measured by ³H-Thymidine incorporation at day 6. Mice were analyzed individually and values shown are the mean ± SEM (n= 3 mice/group). The results are representative of three independent experiments. Statistical significance was evaluated using Student's T test

Recently, it has been shown that antibody activation of CD59 enhances NK

cytotoxicity (Marcenaro *et al.*, 2003). NK cells are critical components in innate

immunity. Downregulation of MHC class I molecules in human cells by VV sensitizes

the cells to lysis by NK cells (Kirwan *et al.*, 2005). Also, vaccinia infection of

macrophages induces release of IL-18 that subsequently activates NK cells (Gherardi

et al., 2003; Tanaka-Kataoka *et al.*, 1999). To test the effect of CD59 deficiency on

NK activity, mice were infected with rVVG2 and NK activity in splenocytes was

measured against YAC- cells at day 3 after infection. Infected mice exhibited increased

NK lytic activity compared to non-infected mice; however, no significant difference

was observed between Cd59-/- and WT mice (Figure 3.3).

3.2.5. Immune Responses in Ovaries and Virus Titres

In order to determine whether the enhanced proliferation of GP-specific CD4$^+$ T cells

observed following *in vitro* stimulation reflected a stronger anti-viral CD4$^+$ T cell

response *in vivo*, numbers of CD4$^+$ T cells at the site of rVVG2 infection (ovary) were

compared in WT and Cd59a -/- mice at day 8 after infection. Higher numbers of

infiltrating CD4$^+$ T cells (Figure 3.4A and C) and IFNγ-producing CD4$^+$ T cells

(Figure 3.4B and D) were observed in the ovaries of Cd59a -/- mice compared to WT

mice. The same analysis was performed for ovary-infiltrating CD8$^+$ T cells (Figures

3.4E and F). Slightly higher numbers of CD8$^+$ T cells were observed in the ovaries of

Cd59a-/- mice compared to WT mice. This difference was not statistically significant

and may reflect enhanced helper CD4$^+$ T cell activity in the Cd59a -/- mice rather than

a direct effect of CD59a on CD8$^+$ T activity. To test whether the higher level of CD4$^+$

T cell activity in the ovaries of Cd59a -/- mice was due to poorer control of virus in

NK

day 3

Legend:
- Cd59a-/-
- WT
- naive Cd59a-/-
- naive WT

Y-axis: % Lysis (0, 10, 20, 30, 40)
X-axis: E:T (200, 66, 22, 8)
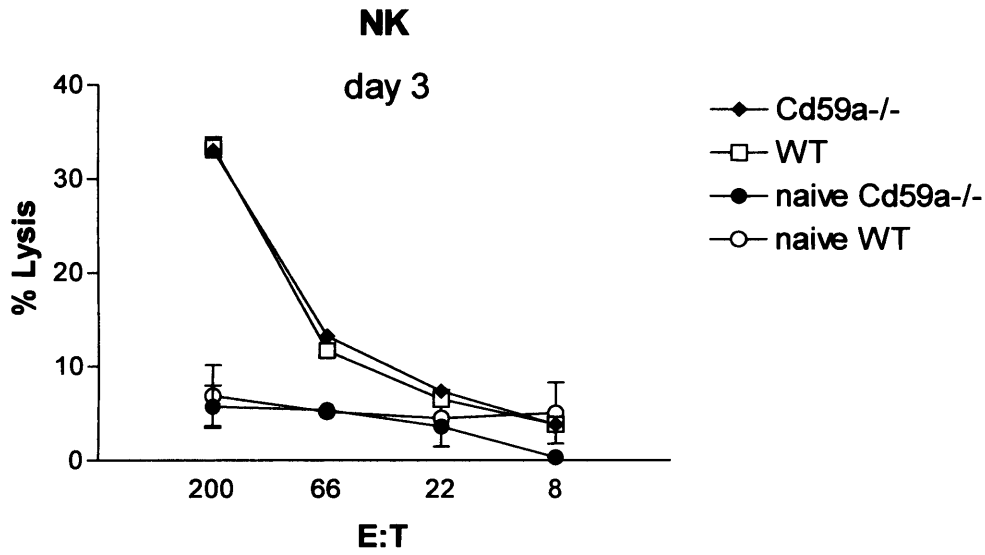
Figure 3.3. Cd59a-/- Mice Have Unaltered NK Cytotoxicity

NK cytotoxic activity was analyzed at day 3 after rVVG2 infection. Spleen cells were incubated with YAC-1 and cell lysis was measured by ^{51}Cr release.
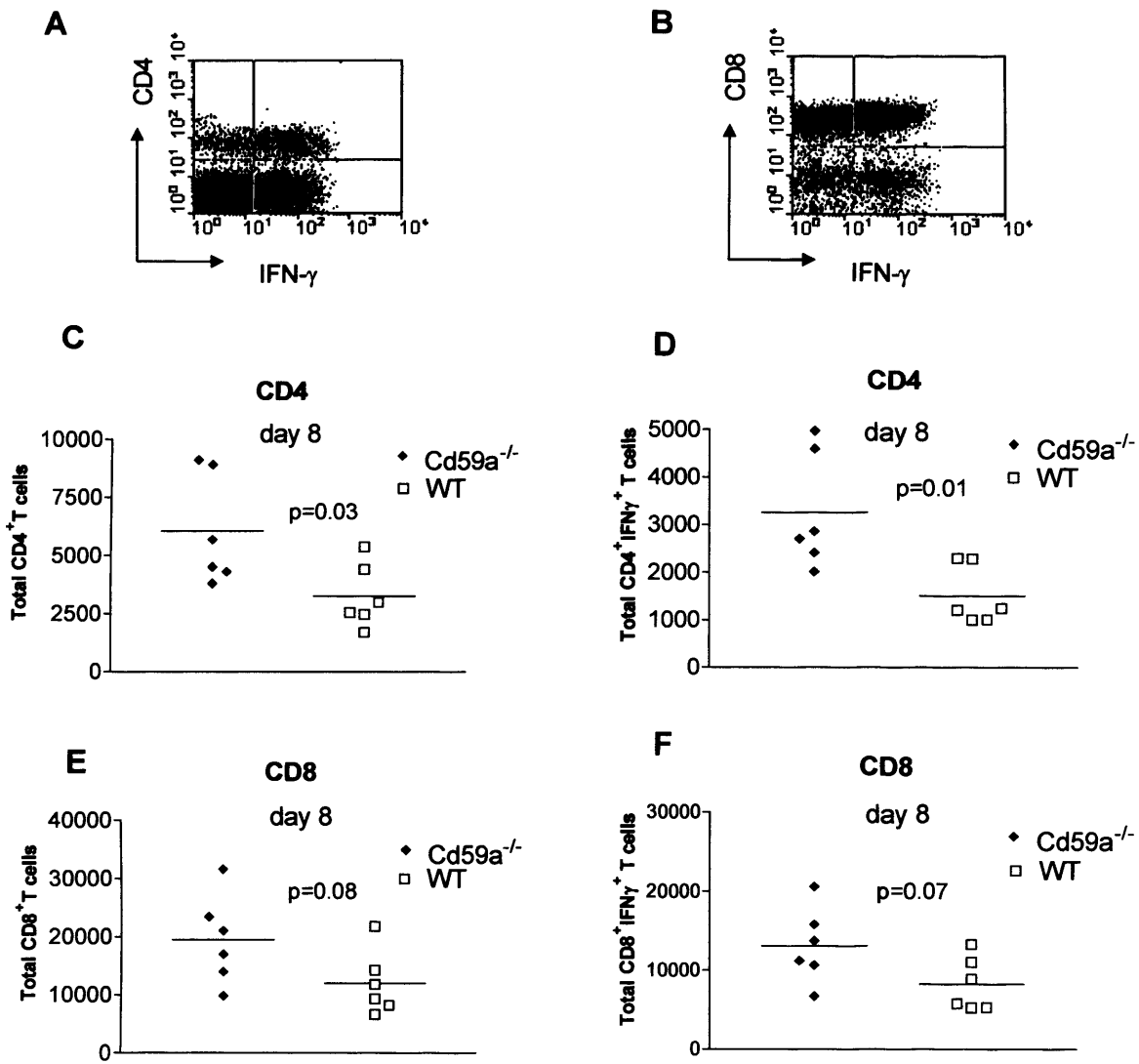
Figure 3.4. Increased Numbers of Ovary-infiltrating CD4⁺ T Cells in Cd59a-/-Mice

Ovary-infiltrating lymphocytes were stained for intracellular IFN-γ, CD4 and CD8 expression at day 8 post-infection and analyzed by FACS. CD4⁺ and CD8⁺ IFN-γ producing cells were gated as shown in A and B respectively. Total numbers of infiltrating and IFN-γ producing CD4⁺ are shown in C and D respectively and total numbers of infiltrating and IFN-γ CD8⁺ T cells are shown in E and F respectively. Each symbol represents an individual mouse and similar data was observed in two independent experiments. Mean titres are also indicated in each graph. Statistical significance was evaluated using Student's t test.
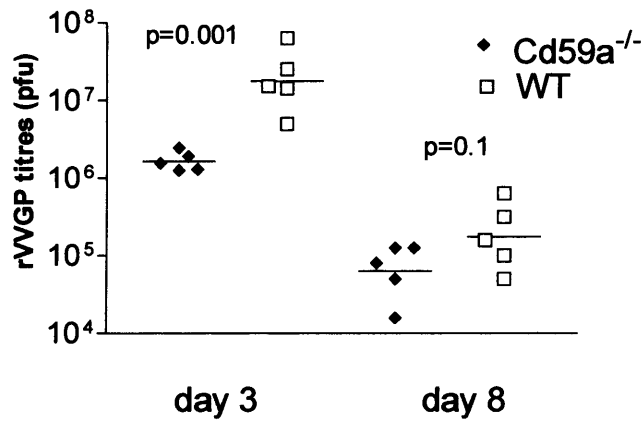
Figure 3.5. CD59a-/- Mice Control rVVG2 Infection More Effectively Compared to WT Mice

Virus titres in the ovaries of infected mice were determined at day 3 and at day 8 post-infection. Virus clearance was more effective in Cd59a-/- mice compared with WT mice at day 3. No significant difference was detected at day 8 post-infection.

these mice, virus titres were compared in both groups of mice at days 3 and 8 post-infection. Virus titres were found to be significantly lower in Cd59a -/- mice compared to WT mice at day 3 post-infection, whereas both groups of mice had controlled the infection by day 8 (Figure 3.5). These data indicate that the more robust CD4$^+$ T cell response observed in Cd59a -/- mice does not reflect an inability to clear the virus efficiently but may rather contribute to more rapid control of the infection.

3.2.6. Analysis of CD4$^+$ T Cell Proliferation *In Vitro*

Proliferation of T cells from WT mice and Cd59a -/- mice was compared following *in vitro* stimulation with CD3-specific mAbs and APCs (T-depleted spleen cells). While no difference was observed in the proliferative behaviour of CD8$^+$ T cells (Figure 3.6A), CD4$^+$ T cells from Cd59a -/- mice exhibited more proliferation *in vitro* compared to T cells from WT mice (a 2.5-fold increase; Figure 3.6B). The increase in proliferation of CD4$^+$ T cells from Cd59a-/- mice compared to WT mice was only apparent when CD3-specific mAb and APCs were used for *in vitro* stimulation and not when CD3- and CD28-specific antibodies were used (compare Figures 3.6B and C). However, it was possible that strong stimulation with CD3- and CD28-specific antibodies could override the effect of CD59. To address this question, CD4$^+$ T cells from Cd59a-/- and WT mice were stimulated with different concentrations of CD3- and CD28-specific antibodies. No difference in CD4$^+$ T cell proliferation between groups was observed at any of the concentrations tested (Figure 3.7).

To determine whether CD59a expression on APCs affected proliferation of the responding T cells, CD4$^+$ T cells were incubated with APCs from either WT or Cd59a -/- mice. CD4$^+$ T cell proliferation was not influenced by the presence or absence of
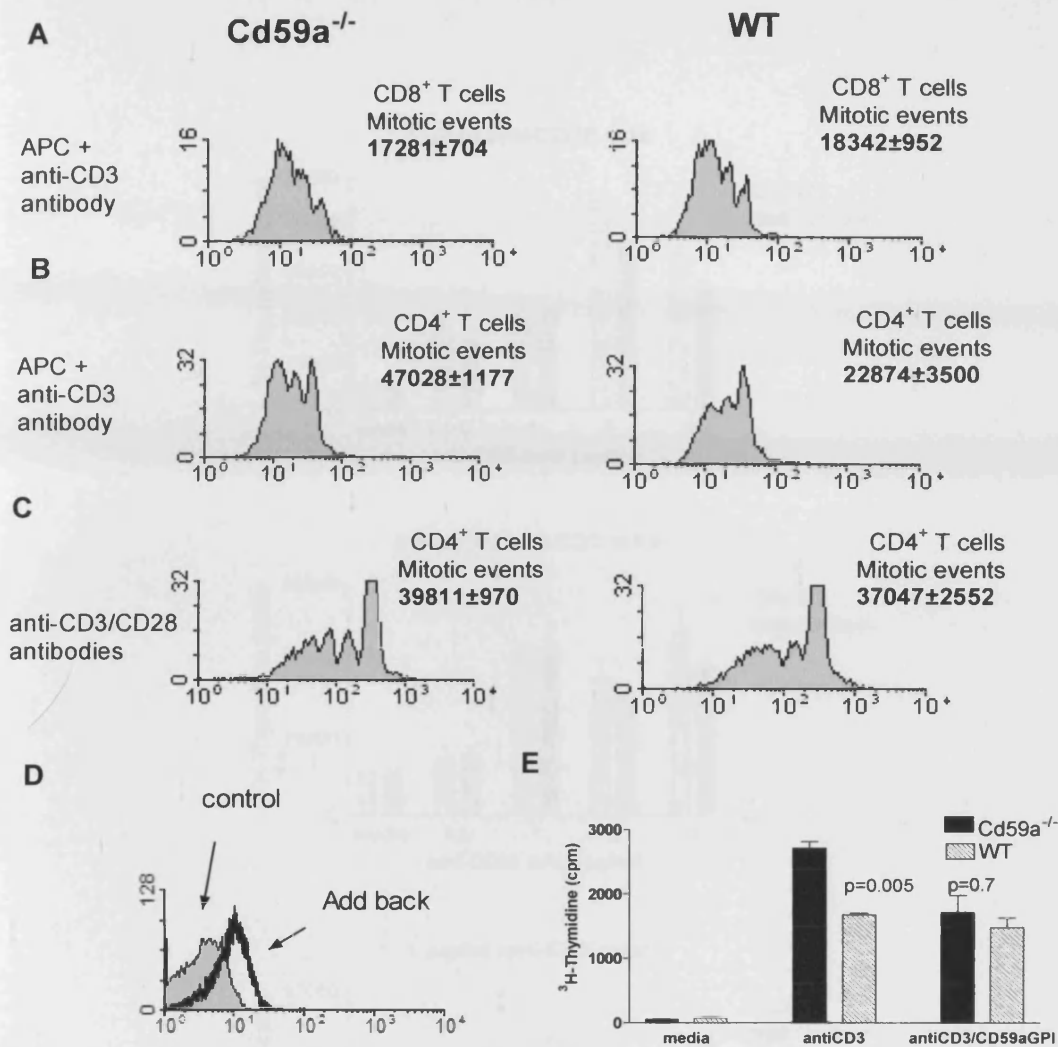
Figure 3.6. *In vitro* **Proliferation Assays**

Purified CD4$^+$ or CD8$^+$ T cells (>90%) were CFSE labeled and incubated with anti-CD3 mAb and APCs (A and B) or anti-CD28 mAb (C). Calculation of the number of mitotic events was performed as previously described (Yamazaki *et al.*, 2003). One representative result of at least three independent experiments is shown. Values indicating mitotic events are the means of three experiments ± SD. To confirm that lack of CD59a expression was responsible for enhanced proliferation of T cells from Cd59a-/- mice, lymphocytes were incubated with GPI-anchored CD59a (CD59a-GPI) before the proliferation assay (E). Incorporation of CD59a-GPI on CD4$^+$ T cells was confirmed by immunostaining (D).Values shown represent the mean ± SD. The experiment was performed on three separate occasions. Statistical analysis was performed by the Student's T test.

2μg/ml anti-CD28 mAb

^{3}H-Thymidine (cpm)

50000
40000
30000
20000
10000
0

media 0.5 1 2 5

anti-CD3 mAb (μg/ml)

WT
Cd59a-/-

0.5 μg/ml anti-CD3 mAb

^{3}H-Thymidine (cpm)

30000
20000
10000
0

Media 0.5 1 2 5

anti-CD28 mAb (μg/ml)

WT
Cd59a-/-

1 μg/ml anti-CD3 mAb

^{3}H-Thymidine (cpm)

30000
20000
10000
0

Media 0.5 1 2 5

anti-CD28 mAb (μg/ml)

WT
Cd59a-/-
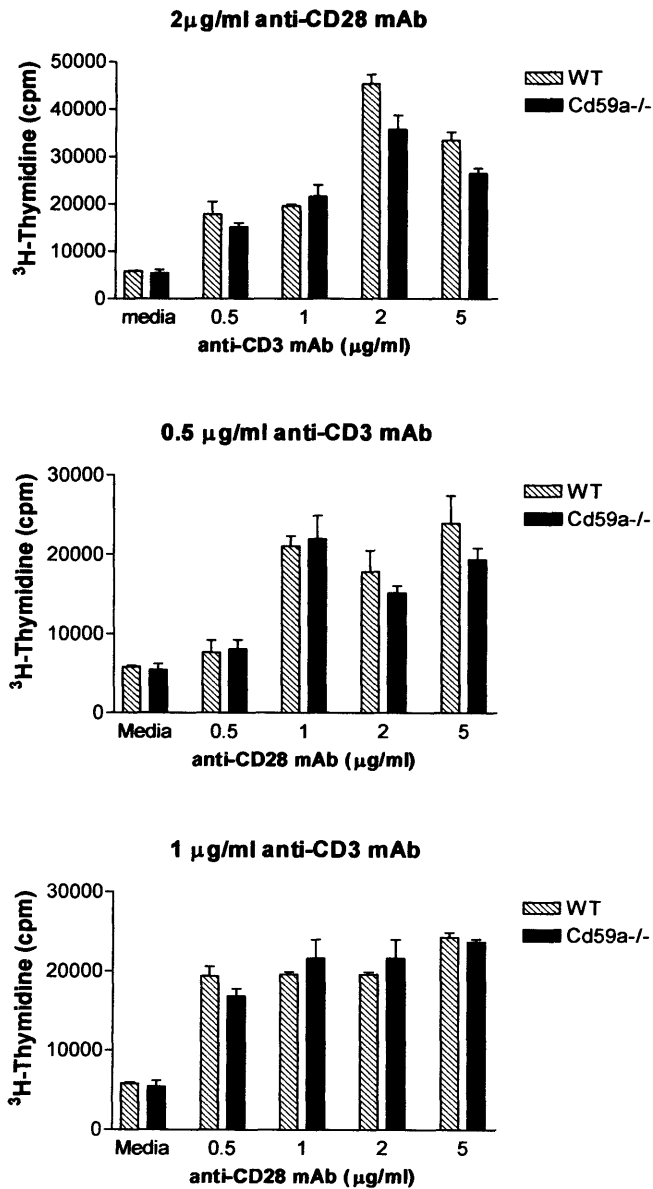
Figure 3.7. Enhanced CD4$^+$T Cell Proliferation Requires The Presence of APCs.

Purified CD4$^+$ T cells from Cd59a-/- or WT mice were stimulated with different concentration of CD3- and CD28-specific antibodies. Proliferation was measured by ^{3}H-Thymidine incorporation. One representative result of two independent experiments is shown. Values shown represent the mean ± SD.

Cd59a-/-
CD4⁺T cells

WT
CD4⁺T cells

APC-CD59a⁺
+ anti-CD3
antibody

Mitotic events
38813± 2252

Mitotic events
21081± 1705

APC-CD59a⁻
+ anti-CD3
antibody

Mitotic events
41104± 1807

Mitotic events
19423± 877
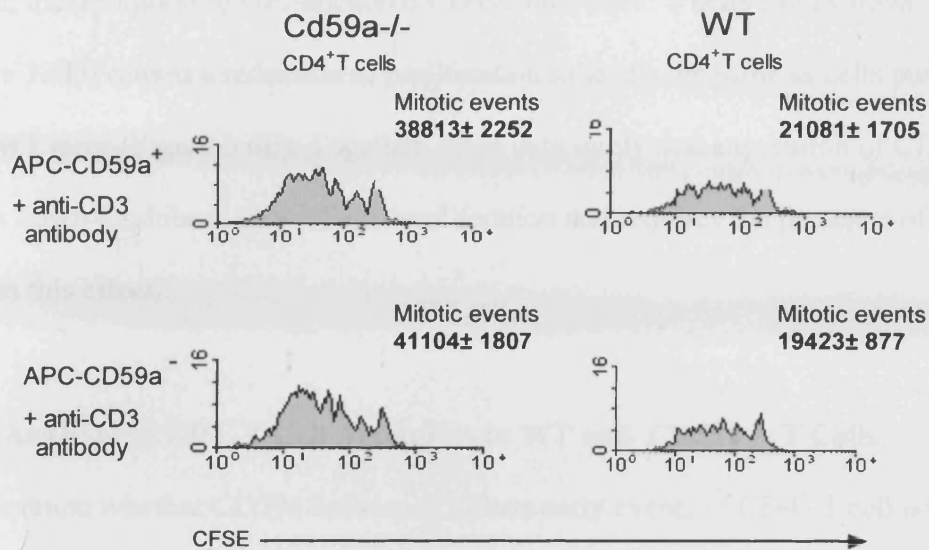
CFSE ⟶

Figure 3.8. Effect of CD59a Expression on APCs
Purified CD4⁺ T cells were CFSE labeled and incubated with anti-CD3 mAb and APCs from Cd59a-/- or WT mice. One representative result of two independent experiments is shown.

CD59a on APCs indicating that the difference in proliferation of CD4$^+$ T cells from WT and Cd59a -/- mice is due to expression of CD59a on the T cells (Figure 3.8). Indeed, incorporation of GPI-anchored CD59a into CD4$^+$ T cells from Cd59a -/- mice (Figure 3.6D) caused a reduction in proliferation to levels the same as cells purified from WT mice (Figure 3.6E). Together, these data imply that expression of CD59a on T cells down-modulates CD4$^+$ T cell proliferation and requires the presence of APCs to exert this effect.

3.2.7. Analyses of CD4$^+$ T Cell Activation in WT and CD59a-/- T Cells

To determine whether CD59a deficiency affects early events of CD4$^+$ T cell activation such as cytokine production, we measured IL-2 levels in CD4$^+$ T cell cultures upon stimulation with CD3-specific antibodies and APCs (Figure 3.9). CD4$^+$ T cell culture supernatants from Cd59a-/- mice showed a modest increase in IL-2 levels after 4 hours of stimulation compared to culture supernatants from WT T cells (Figure 3.9A). The difference became more significant after 18 hours of stimulation (Figure 3.9B). Higher levels of secreted IL-2 could reflect greater numbers of activated cells in cultures of CD59a-/- T cells. To address this question, we analyzed IL-2 production on a per cell basis by intracellular staining. CD4$^+$ T cells from Cd59a-/- mice secreted more IL-2 per cell compared to WT T cells as determined by mean fluorescence (MF; Figure 3.9C). These data demonstrate a consistent correlation between the degree of IL-2 production and proliferative responses observed in CD4$^+$ T cells from Cd59a-/- and WT mice.

In order to further analyse T cell activation events, expression of markers associated with T cell activation was compared in WT and CD59a -/- CD4$^+$ T cells.
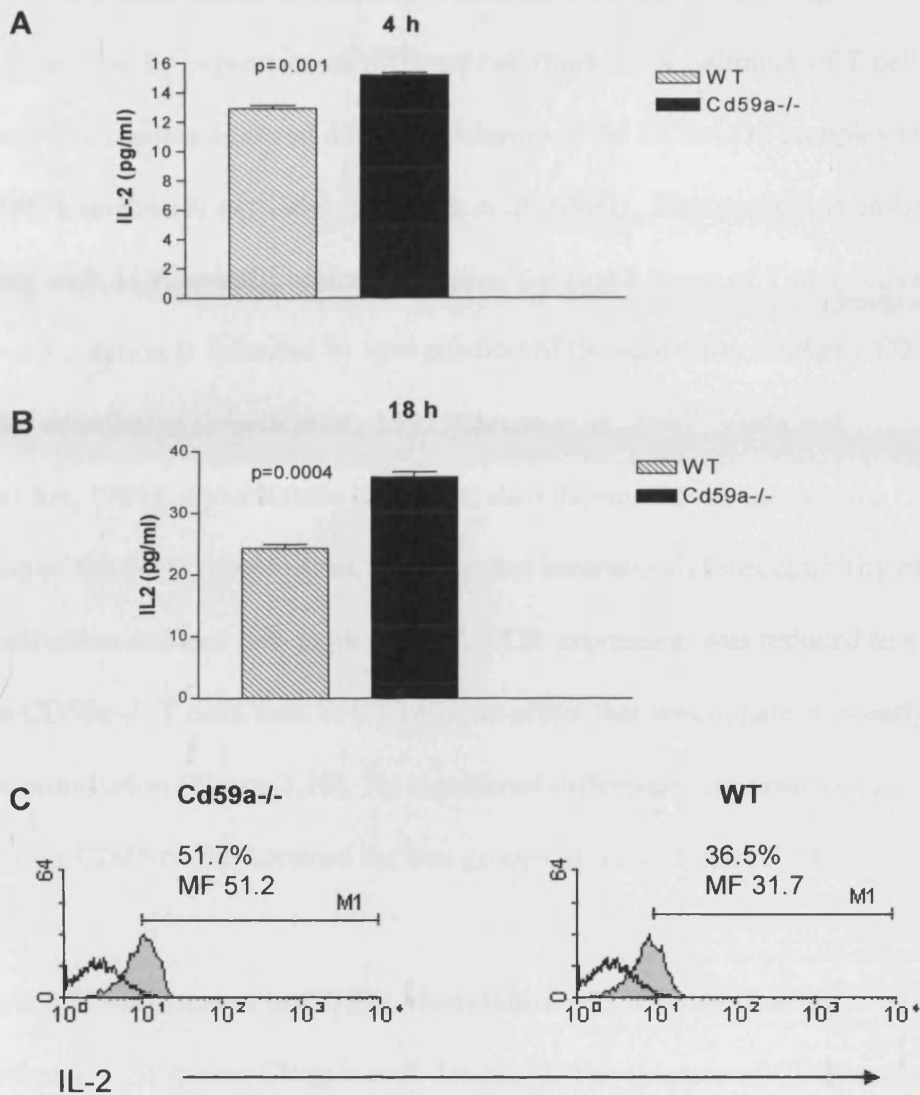
Figure 3.9. Increased Release of IL-2 in T cells From Cd59a-/- Mice Upon Activation

Purified CD4$^+$T cells were activated with CD3-specific antibodies in the presence of APCs. Supernatants were taken at 4 (A) and 18 (B) hr after activation and IL-2 was detected by ELISA. Levels of IL-2 produced per cell were analyzed by intracellular staining of cells after 18 hr of activation (C). Markers were set using isotype staining controls.

Stimulation of T cells results in a number of biochemical and morphological changes that can be tracked by expression of different cell markers. A hallmark of T cell activation is the ligation-induced down-modulation of the TCR:CD3 complex (Boyer *et al.*, 1991; Lauritsen *et al.*, 1998; Telerman *et al.*, 1987) . This process is easily seen by staining with TCR-specific antibodies within the first 5 hours of T cell activation. TCR downregulation is followed by upregulation of the activation marker CD25 24-48 hours after stimulation (Biselli *et al.*, 1992; Caruso *et al.*, 1997; Viola and Lanzavecchia, 1996). Around three days after stimulation, T cells upregulate expression of the death receptor Fas, an event that increases the susceptibility of the cells to activation induced cell death (AICD). TCR expression was reduced to a greater extent on CD59a -/- T cells than WT T cells, an effect that was apparent as early as 1 hour post-stimulation (Figure 3.10). No significant difference was observed in expression of CD25 or Fas between the two groups of mice (Figure 3.11).

3.2.8. Role of Complement in CD59a Modulation of T Cell Activation

The function of CD59a as a CReg is well described. The absence of CD59a allows formation of the MAC and renders the cell more susceptible to complement-mediated cell lysis. As described above, expression of CD59a appears to modulate T cell activity. To test the possibility that C activation and formation of the MAC mediates this effect, CD4$^+$ T cells purified from both WT and Cd59a-/- mice were stimulated with CD3-specific antibodies and APCs in the presence and absence of a soluble inhibitor of complement (sCR1). sCR1 blocks activation of the classical and the alternative pathways of complement by binding C3b and C4b and mediating proteolytic degradation of these molecules (Kalli and Fearon, 1994). Inhibition of complement *in vitro* did not affect the proliferative response of Cd59a-/- T cells

A

Cd59a-/-

WT

TCR

- — 0 h
- — 1 h
- — 3 h
- — 5 h
- — 18 h
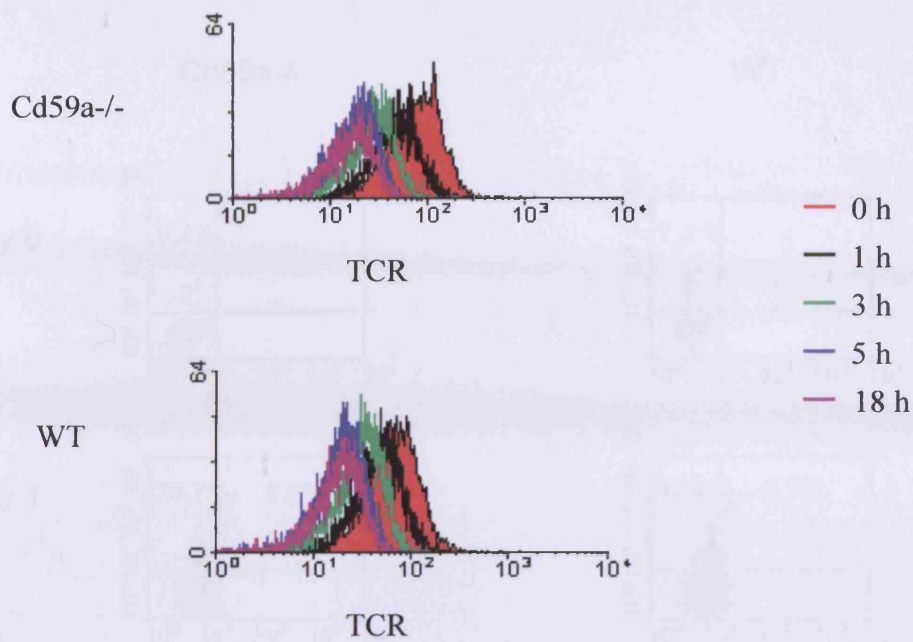
B

TCR Expression (MF)

	0 h	1 h	3 h	5 h	18 h
Cd59a-/-	91.4 ± 6.2	52.6 ± 3.1	36.7 ± 1.3	21.8 ± 2.2	22.1 ± 1.3
WT	90.2 ± 7.5	68.2 ± 4.8	42.9 ± 1.9	29.9 ± 1.7	30.8 ± 0.9

Figure 3.10. TCR Down-regulation Upon Activation
Purified CD4$^+$T cells were activated with CD3-specific antibodies in the presence of APCs (n=3). Lymphocyte activation was analyzed by down-regulation of TCR at different time points. (A) represent a representative histogram for each group. Table in (B) shows the MF of TCR expression on CD4$^+$ T cells. Values shown represent the mean ± SD.
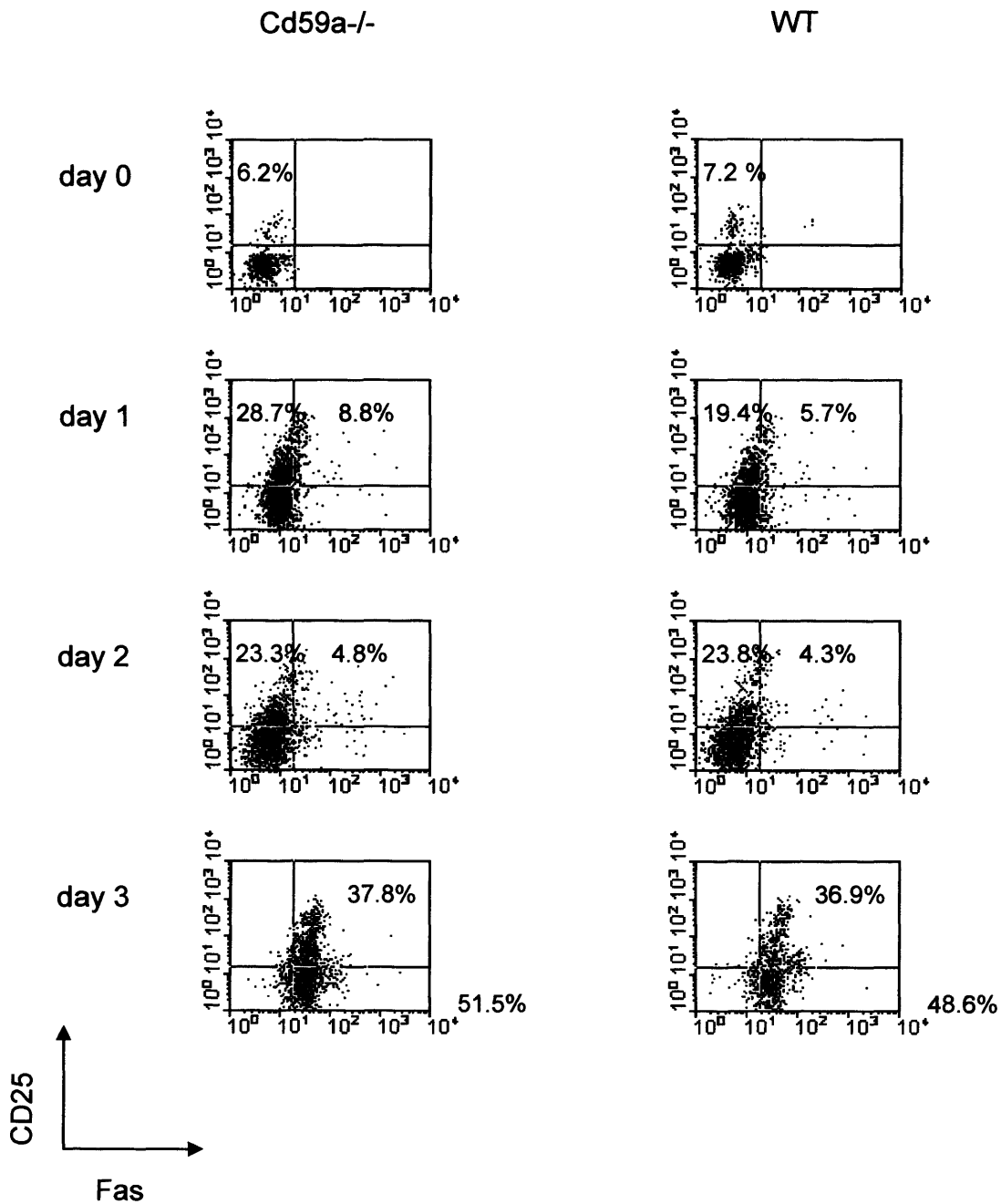
Figure 3.11. CD4⁺T Cell Activation

Purified CD4⁺T cells were activated with CD3-specific antibodies in the presence of APCs. Lymphocyte activation was analyzed by expression of CD25 and Fas every 24 hr for the period of 3 days.

indicating that the enhanced proliferation was complement independent (Figure 3.12A). To determine whether this was also true *in vivo*, mice infected with rVVG2 were injected daily with sCR1. Treatment of mice with sCR1 was shown to efficiently inhibit complement activity (more than 85% inhibition) throughout the course of the experiment. Glycoprotein-specific CD4^{+} T cell responses were measured 9 days after infection and no difference was observed in virus-specific proliferative responses induced in the presence or absence of complement inhibition (Figure 3.12B). To confirm this result, Cd59a-/- mice were intercrossed with C3-/- mice and then infected with rVVG2. Virus-specific CD4^{+} T cell response were enhanced equally in Cd59a-/- and Cd59a-/- C3-/- mice compared to WT and C3-/- mice (Figure 3.12C). These data demonstrate that the enhanced proliferation of Cd59a-/- CD4^{+} T cells is complement independent.
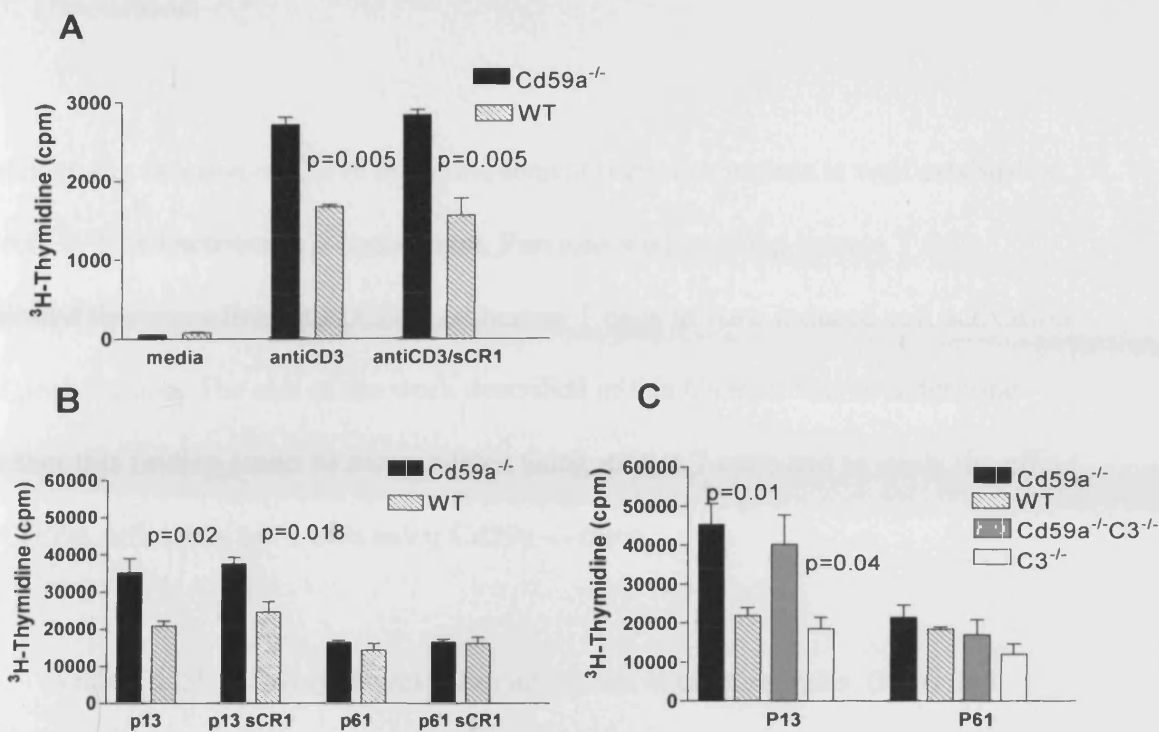
Figure 3.12. Role of C in CD4$^+$ T cell proliferation

Complement was inhibited *in vitro* with sCR1 (i.v.) in assays where CD4$^+$ T cells were stimulated with CD3-specific mAb and APCs (A). Data is representative of two experiments and values are shown as mean ± SD. C was inhibited *in vivo* by administration of sCR1 to mice for the first 9 days of infection with rVVGP (B). *In vivo* experiments were repeated with Cd59a$^{-/-}$C3$^{-/-}$ mice (C). Mice were individually analyzed and the values shown indicate the mean ± SEM (n= 3 mice/group). The results were analyzed statistically by Student's t test

3.3. Discussion

Although the function of CD59 as a complement regulator protein is well established, its role in T cell activation is less certain. Previous studies using human T cells indicated that cross-linking of CD59 on human T cells *in vitro* induced cell activation and proliferation. The aim of the work described in this Chapter was to determine whether this finding could be recapitulated using mouse T cells and to study the effect of CD59a deficiency on T cells using Cd59a -/- mice.

Whilst CD59 is highly expressed in all human circulating cells (Meri and Lachmann, 1991; Nose *et al.*, 1990), expression of CD59a on mouse lymphocytes is low but detectable by WB and RT-PCR. Cross-linking of CD59a on T cells failed to induce T cell activation measured by IL-2 release, probably as a consequence of low levels of CD59 expression. Indeed, in order to reproduce the cross-linking experiments previously performed using human T cells, mouse T cell lines had to be transfected in order to increase the expression level of CD59a. In this chapter, we took advantage of mice deficient for the human homologue of CD59 (CD59a), to better understand the effect of CD59 in T cell activation *in vivo* and *in vitro*.

In order to gain more insight into the effect of CD59a on T cell activation, Cd59a -/- and WT mice were infected with a rVV expressing the GP of LCMV. Cd59a-/- mice demonstrated significant increases in CD4$^+$ T cell proliferation to rVVG2 compared to WT. This was not due to a poor control of the virus since VV

titres measured in the ovary after day 3 post-infection were reduced compared to WT mice.

CD4$^+$ T cells have been shown to play a crucial role in clearance of vaccinia virus by both humoral and cell-mediated mechanisms (Xu *et al.*, 2004b). CD4$^+$ T cells provide the cognate signals to B cells for production of protective virus-neutralizing IgG and, upon activation, they produce IFN–γ which exhibits a direct anti-viral effect (Belyakov *et al.*, 2003; Galmiche *et al.*, 1999; Harris *et al.*, 1995; Karupiah *et al.*, 1993). The high numbers of CD4$^+$ T cells infiltrating the ovaries of Cd59a-/- infected mice parallels the increase in proliferation of the virus-specific CD4$^+$ T cells, resulting in increased local production of IFN-γ and more efficient virus clearance.

An alternative explanation for enhanced CD4$^+$ T cell responses in Cd59a-/- mice involves complement. CD59a protects cells from complement mediated cell lysis inhibiting the formation of the MAC. Infection with rVVG2 may lead to complement activation that will be amplified in the absence of CD59a. Formation and deposition of MAC could induce cell activation as previously shown with mesangial cells (Lovett *et al.*, 1987). However, our data demonstrate that the enhanced proliferation of Cd59a -/- CD4$^+$ T cells is complement independent. *In vivo* inhibition of complement with sCR1 did not alter rVV-specific CD4$^+$ T cell proliferation in Cd59a-/- mice responses remained higher than in WT mice. To confirm this result, Cd59a-/- mice were back-crossed into C3-/-mice. VV-infection of Cd59a-/- C3-/- mice induced increased virus-specific CD4$^+$ T cell activity similar to Cd59a-/- mice.

Finally, in contrast to the cross-linking experiments performed *in vitro*, these data indicate that CD59a downmodulates anti-viral $CD4^+$ T cell responses induced *in vivo*. These data are similar to those reported recently using CD55-deficient mice (Daf1-/- mice) where lymphocytes from these mice were found to proliferate more vigorously in response to a range of antigens compared to wild-type mice (Heeger *et al.*, 2005; Liu *et al.*, 2005). However, enhanced proliferation of Cd59a -/- $CD4^+$ T cells is complement independent, contrasting with the recent data reported for Daf1-/- mice (Heeger *et al.*, 2005; Liu *et al.*, 2005). In the case of CD55, the investigators found that the enhanced proliferative responses observed in Daf1-/- mice were largely, although not exclusively, complement dependent. Thus, while CD55 affects T cell activity in both a complement dependent and independent fashion, this study suggests that the effect of CD59a is complement independent. Another study reported that T cells isolated from Ly-6A – deficient animals also proliferate more vigorously than those isolated from their wild-type counterparts (Stanford *et al.*, 1997). Interestingly Ly-6A, CD59 and CD55 are all GPI-anchored molecules localized in lipid rafts, and all have previously been found in cross-linking studies to promote T cell activation *in vitro* (Davis *et al.*, 1988b; Korty *et al.*, 1991; Stanford *et al.*, 1997). Despite this, all three molecules negatively regulate T cell activity *in vivo*. Since GPI-linked proteins weakly associate with protein tyrosine kinases, it is possible that they can act either as positive or negative regulators of T cell activation by sequestering signalling molecules or interfering with assembly of signalling complexes in lipid rafts, the net effect dependent upon the trigger.

In vitro

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Significantly, *in vitro* studies of T cell responses in Cd59a-/- mice recapitulate the *in vivo* data. Stimulation of CD4$^+$ T cells with CD3- specific antibodies and APCs induced increased cell proliferation in CD59a-/- compared to WT, though they responded in a similar way to CD3- and CD28- specific stimulation. These data suggest the presence of a ligand on APCs which, upon engagement with CD59a, down-modulates CD4$^+$ T cell proliferation. APCs have been shown to express different complement component that may lead to MAC formation (Morgan and Gasque, 1997). Inhibiting complement had no effect on the enhanced proliferation observed in CD59a -/- T cells indicating that the ligand is not part of the complement cascade. The nature of the ligand is uncertain although several investigators have proposed that the natural ligand for human CD59 is CD2 (Deckert *et al.*, 1992; Hahn *et al.*, 1992). In one study, it was shown that radiolabelled CD59 bound to CD2-transfected CHO cells and that CD2-specific antibodies blocked binding (Deckert *et al.*, 1992). Using surface plasmon resonance analyses however, no interaction could be detected between the two molecules (Van der Merwe *et al.*, 1994). The identity of a natural, non-complement ligand for CD59a will be further discussed in Chapter 5.

TCR engagement triggers a series of events, including early events such as TCR down-regulation, cytokine release, and late events such as proliferation. The results presented in this Chapter indicate that both early and late events are affected by CD59a. Activated CD4$^+$ T cells lacking the expression of CD59a produce higher levels of IL-2 compared to WT CD4$^+$ T cells and show more rapid and extensive TCR down-regulation. The biological significance of TCR downregulation is still unclear. TCR ligation is associated with receptor downregulation and this dynamic regulation of TCR cell surface expression is thought to be an important mechanism for T cells to

calibrate their response to different levels of stimuli. A more rapid TCR

downregulation may be a consequence of a lower activation threshold on CD59a-

deficient CD4$^+$ T cells or may protect the cells from over-stimulation and anergy

induction. It has been proposed that TCR downregulation is essential for T cell

activation (Valitutti *et al.*, 1995; Viola and Lanzavecchia, 1996). TCR internalization

may serve as a focus for kinase association promoting or facilitating intracellular

signaling (reviewed in Rothenberg, 1996). An alternative explanation for TCR

downregulation is that internalization of the receptors limits prolonged contact with

antigen, and thereby prevents tolerance by excessive TCR signalling. Together, these

data suggest that CD59a modulates the early events of T cell activation.

In summary, the experiments described in this chapter identify a role for the

GPI-anchored complement regulator protein, CD59a, in negative modulation of T cell

activity *in vivo and in vitro*. Some key questions arise from this data. Firstly, is

negative modulation of T cells by CD59a important for limiting the extent of T cell

proliferation and therefore any pathological consequences of a T cell response?

Secondly, what is the mechanism through which CD59a exerts its T cell modulating

effect? Lastly, is the effect of CD59 on human T cells similar to that observed on

mouse cells? These questions will be addressed in the following three chapters.

Chapter 4 – Influenza-induced immunopathology in Cd59a-/- mice

4.1. Introduction

Influenza virus infection represents a significant health problem, causing high morbidity and mortality worldwide. As described in detail in Chapter 1, influenza virus infection induces a massive pulmonary inflammatory response involving cells of the innate and adaptive immune system that can result in lung pathology. In studies of asthma in mice, complement activation has been shown to promote infiltration of inflammatory cells into the lungs (Krug *et al.*, 2001; Marc *et al.*, 2004). In addition, lack of complement component C3, results in reduced T cell infiltration into the lungs of influenza-infected mice. These findings imply that complement activation could contribute to virus-induced immunopathology and it is possible that the protective effects of CReg such as CD59a limit this process. The results presented in the previous chapter showed that CD59a modulated the $CD4^+$ T cell response to rVV in a complement-independent manner. It is possible therefore that CD59 also modulates the $CD4^+$ T cell response following infection with influenza virus, thereby limiting the extent of T cell-mediated immunopathology in a complement independent fashion. Based on this information, it was hypothesized that a deficiency in Cd59a would result in enhanced innate and adaptive immune responses to influenza virus and therefore enhanced immunopathology, mediated by complement dependent and independent mechanisms. This chapter describes experiments that address this hypothesis.

4.2. Results

4.2.1. Cd59a-/- Mice Exhibit Increased Lung Inflammation Compared to WT Mice

Experimental influenza infection results in significant lung injury that can be detected by histological study of infected lungs. Cd59a-/- and WT mice were infected with influenza virus (H17, H3N2) (Townsend and Skehel, 1984) intra-nasally and lungs were evaluated for evidence of pathology at day 8 post-infection. H&E stained sections of a non-infected lung and influenza-infected lungs of Cd59a-/- and WT mice are shown in Figures 4.1A-C. Lung pathology was scored by an independent observer who examined the lungs for evidence of alveolar exudates, (score 0 to 3); haemorrhages (score 0 to 4); interstitial inflammatory infiltrate (macrophages and lymphocytes, score 0 to 4) and fibrosis (score 0 to 5) (see Figure 4.1D). Low levels of alveolar exudates were observed in both groups of mice. Areas of haemorrhage and areas of inflammatory infiltration were more extensive in Cd59a -/- mice compared to WT mice. Areas of fibrosis were observed only in Cd59a-/- mice. Each score were added together for every individual mouse to compile a final histological score (0-16). As shown in Figure 4.1E, the final histological score, and therefore the extent of lung pathology was significantly greater in Cd59a -/- mice.

4.2.2. Innate Immune Response to Influenza Virus

A- Normal Lung

Alveolar epithelium

Bronchiole

Alveolus
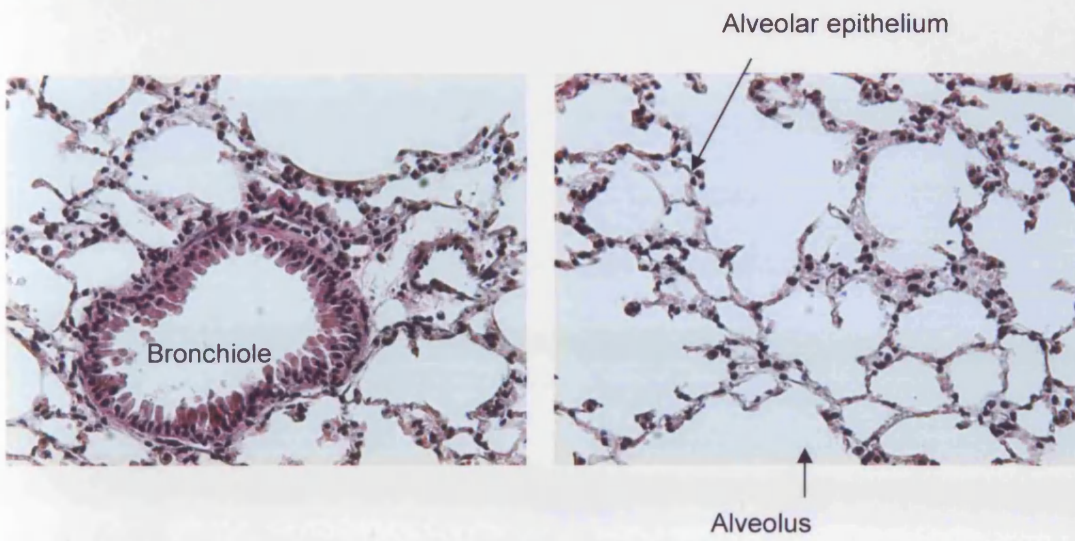
B- Lung from WT Flu-infected mouse
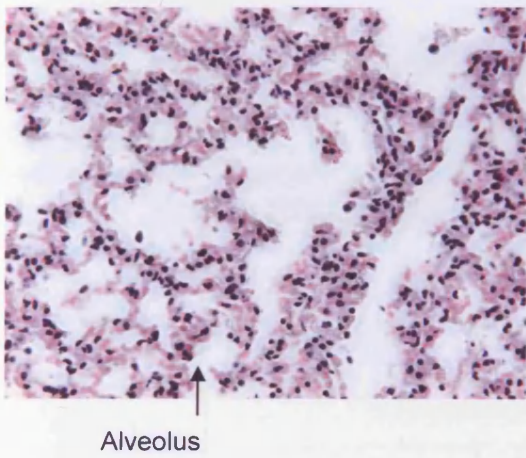
C- Lung from Cd59a-/- Flu-infected

Alveolus

Alveolus

D   **E**

0-4

5-10

11-16
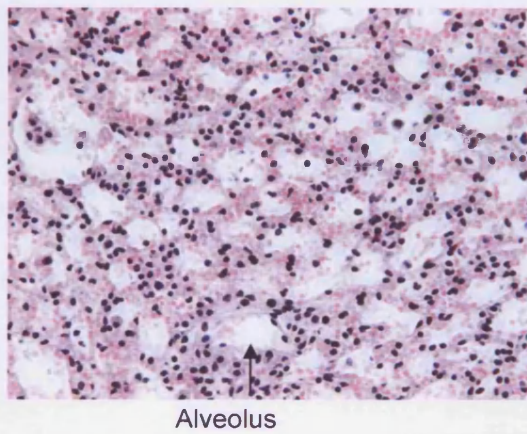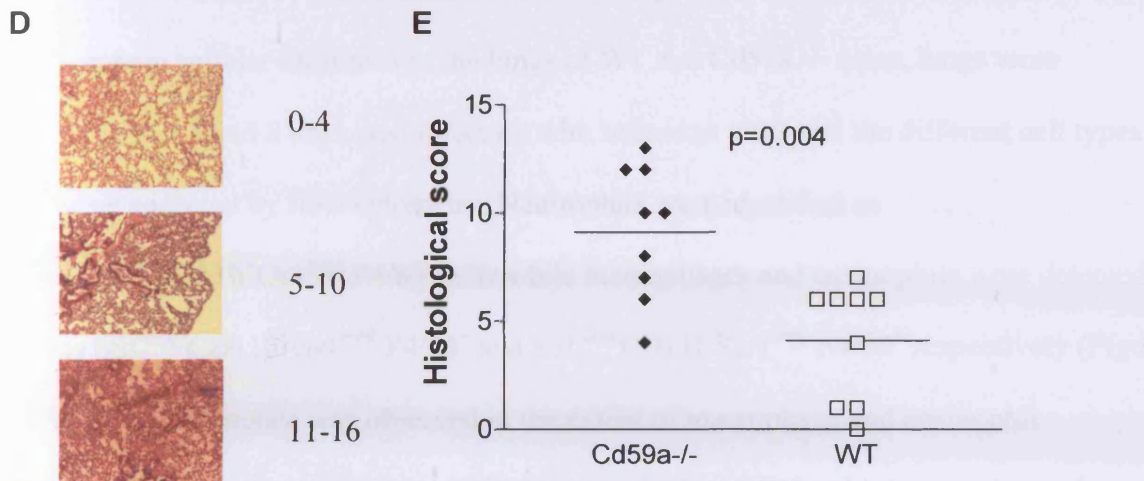
Figure 4.1. Cd59a-/- Mice Exhibit Increased Lung Inflammation Compared to WT Mice

Mice were infected with influenza virus and lungs removed 8 days after infection (n=6/ group). (A) represent a healthy lung. Lungs from Cd59a -/- (B) or WT (C) mice were fixed in Zinc fixative, sectioned and staining with H & E. Representative histological score of lungs H&E staining are shown in (D). Histological score was evaluated by an independent observer (E). Statistical significant was evaluated by Mann-Whitney U test.

Several reports indicate that the extent of influenza-induced lung pathology correlates

with the extent of cellular infiltration in the lungs of infected mice. In order to

compare cellular infiltrates in the lungs of WT and Cd59a -/- mice, lungs were

harvested 3 and 8 days post infection with influenza virus and the different cell types

were analysed by flow cytometry. Neutrophils were identified as

$SSC^{high}CD11b^{+}Gr1^{high}$ F4/80^{-} cells while macrophages and eosinophils were detected

as $SSC^{high}CD11b^{+}Gr1^{med}$ F4/80^{+} and $SSC^{low}CD11b^{+}Gr1^{med}$ F4/80^{+} respectively (Figure

4.2). No difference was observed in the extent of macrophage and eosinophil

recruitment between groups of mice (Figure 4.3A and B) but higher numbers of

neutrophils were observed in the lungs of Cd59a-/- compared to WT mice (Figure

4.3C).

NK cells are activated in response to infection with influenza virus (see

Chapter 1). In order to assess NK cytotoxicity, Cd59a-/- and WT mice were infected

with influenza virus and a killing assay was performed at day 3 post-infection. The NK

cytolytic activity against YAC-1 cells was low but similar for both groups of mice

(Figure 4.4A). Total numbers of NK cells recruited to infected lungs was analysed by

flow cytometry at day 3 and 8 after infection. High numbers of NK cells were found in

lungs of infected mice but numbers were similar between Cd59a-/- and WT mice

(Figure 4.4B)

4. 2.3. Cd59a-/- Mice Exhibit Increased Numbers of Lung-Infiltrating

Lymphocytes after Influenza Infection

Influenza infection of mice induces a rapid release of cytokines by infected cells and

recruitment of lymphocytes within the lung (Enelow *et al.*, 1998). Total numbers of
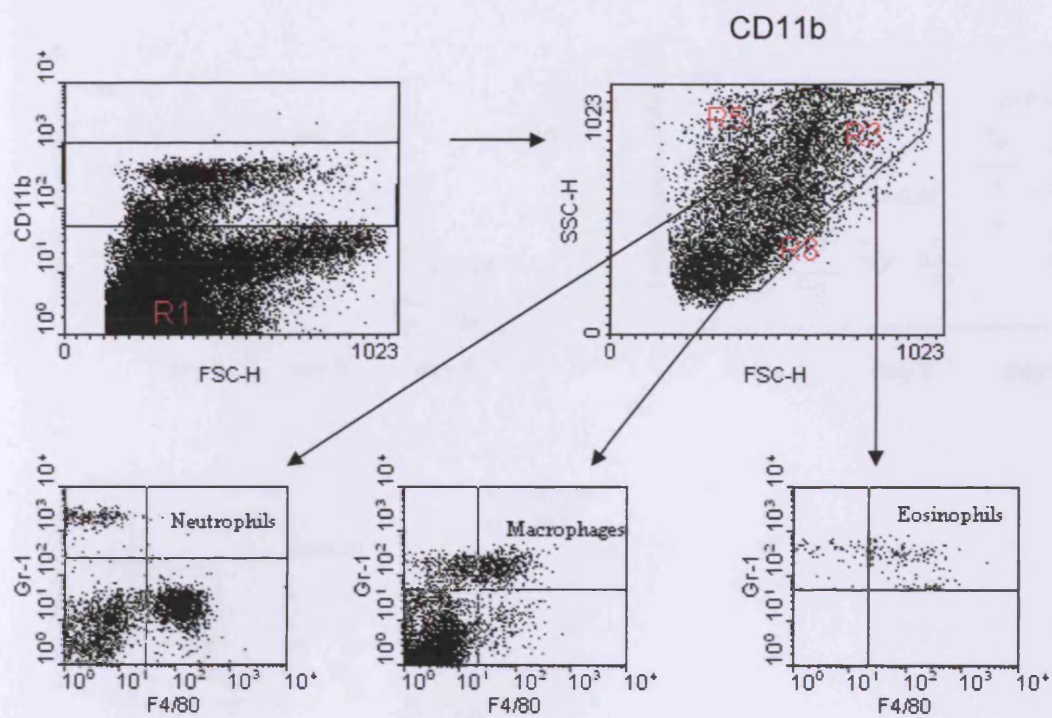
Figure 4.2. Identification of Neutrophil, Eosinophil and Macrophage populations
Presence of neutrophils, eosinophils and macrophages in influenza-infected lungs was
analyzed by flow cytometry. Cells were identified by forward (FSC) side (SSC) scatter
and antibody binding to CD11b, Gr-1 and F4/80: $SSC^{high}CD11b^{+}Gr1^{high}$ $F4/80^{-}$
(neutrophils), $SSC^{high}CD11b^{+}Gr1^{med}$ $F4/80^{+}$ (eosinophils) and $SSC^{low}CD11b^{+}Gr1^{med}$
$F4/80^{+}$ (macrophages).

A

Total Macrophages

p=0.26

p=0.93

p=0.28

◆ Cd59a-/-
□ WT

day 0   day 3   day 8

B

Total Eosinophils

p=0.43

p=0.77   p=0.57

◆ Cd59a-/-
□ WT

day 0   day 3   day 8

C

Total Neutrophils

◆ p=0.01

p=0.83

p=0.46

◆ Cd59a-/-
□ WT

day 0   day 3   day 8
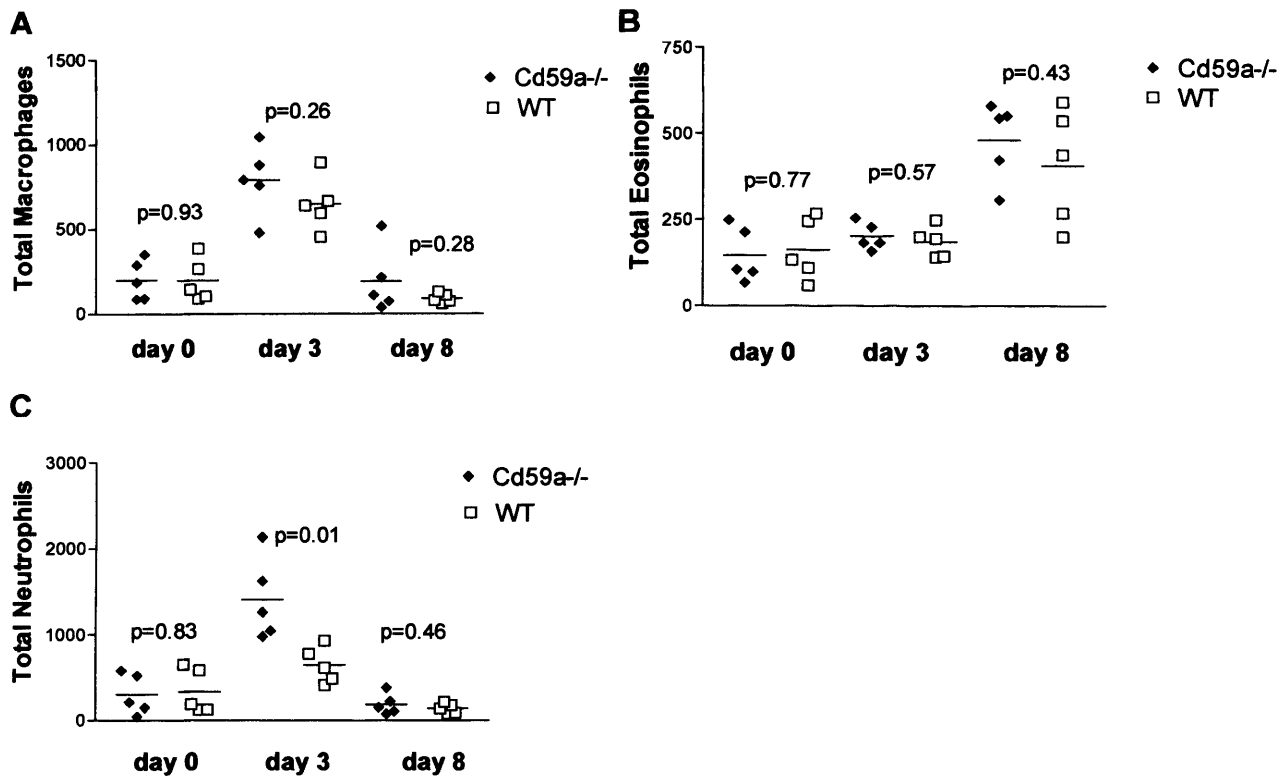
Figure 4.3. Increased Neutrophil Recruitment into Lungs of Cd59a-/- Mice
Mice were infected with influenza virus and lung-infiltrating macrophages (A),
eosinophils (B) and neutrophils (C) were analyzed by flow cytometry as explained in
Figure 4.2. Each symbol represents an individual mouse and similar data was observed
in two independent experiments. Means are also indicated in each graph. Statistical
significance was evaluated using Student's t test.
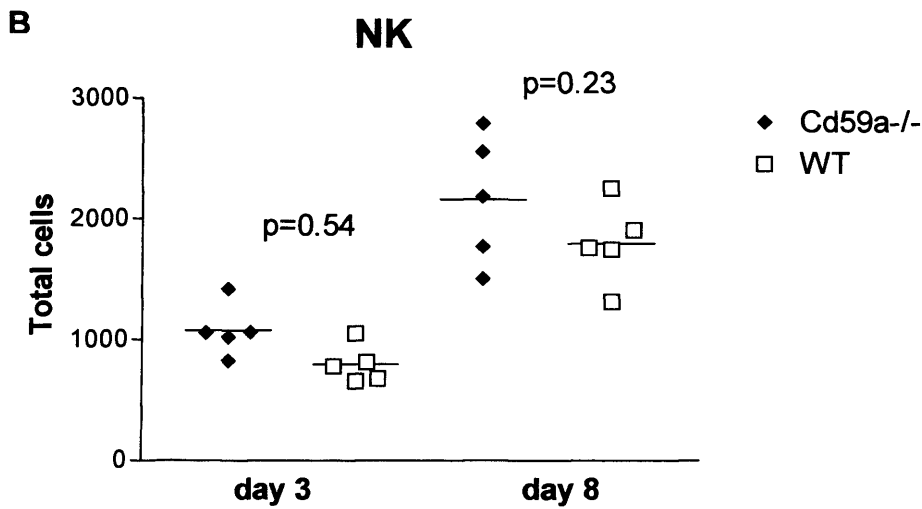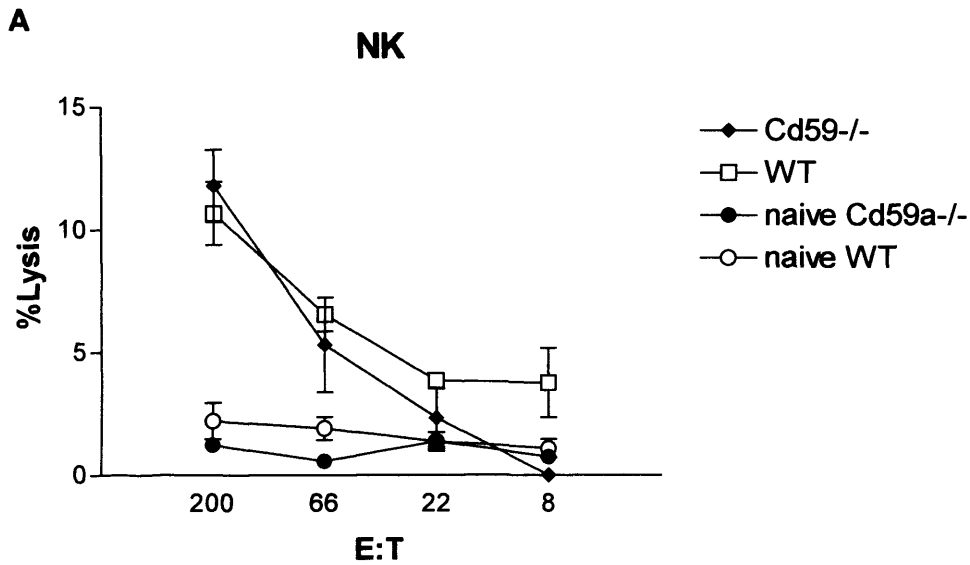
Figure 4.4. Cd59a-/- Mice Have Unaltered NK Cytotoxicity
NK cytotoxic activity was analyzed at day 3 after Influenza virus infection. Spleen cells were incubated with YAC-1 and cell lysis was mesured by ^{51}Cr release (A). Total numbers of lung-infiltrated NK cells were analyzed by flow cytometry (B). Mice were analyzed individually and values shown are the mean ± SEM (A) and each symbol represents an individual mouse (B). Similar data was observed in two independent experiments. Statistical significance was evaluated using Student's t test.

lung-infiltrating lymphocytes were analysed by flow cytometry at day 3, 8 and 12 post-infection. Both, Cd59a-/- and WT mice showed a maximal T cell expansion at day 8 post-infection that decreased significantly by day 12 (Figure 4.5A and B). The number of CD4$^+$ T cells recovered from the lungs at day 3 post-infection was strikingly higher in Cd59a-/- compared to WT mice. Similar results were observed at day 8 post-infection and by day 12, numbers of CD4$^+$ T cells in lungs were similar for both groups of mice (Figure 4.5A). Total numbers of CD8$^+$ T cells infiltrating the lungs were reduced compared to CD4$^+$T cells peaking at day 8 post-infection. Slightly higher numbers of CD8$^+$ T cells were observed in the lungs of Cd59a-/- mice compared to WT mice but the difference between the two groups was not statistically significant except at day 12 post-infection (Figure 4.5B). The trend for higher numbers of CD8$^+$ T cells in the lungs of Cd59a-/- mice compared to wt mice may reflect enhanced helper CD4$^+$ T cell activity in the Cd59a -/- mice (Figure 4.5B). Antigen-specific CD8$^+$ T cell numbers in the lungs of both groups of mice was also analysed on days 8 and 12 post-infection by tetramer staining for the inmunodominant peptide np68. No significant difference was observed between the mouse groups (Figure 4.5C).

Functional assays were performed to assess the activity of the infiltrating lymphocytes in Cd59a -/- and wt mice. IFN-γ production by T cells recovered from the lungs was assessed by intracellular staining and flow cytometry following stimulation of the cells with PMA/ionomycin. IFN-γ-producing cells were mainly detected at day 8 post-infection. The number of IFN-γ producing CD4$^+$ T cells in Cd59a-/- mice was significantly higher than WT mice (Figure 4.5D). No difference was found in the number of IFN-γ-producing CD8$^+$ T cells (Figure 4.5E).
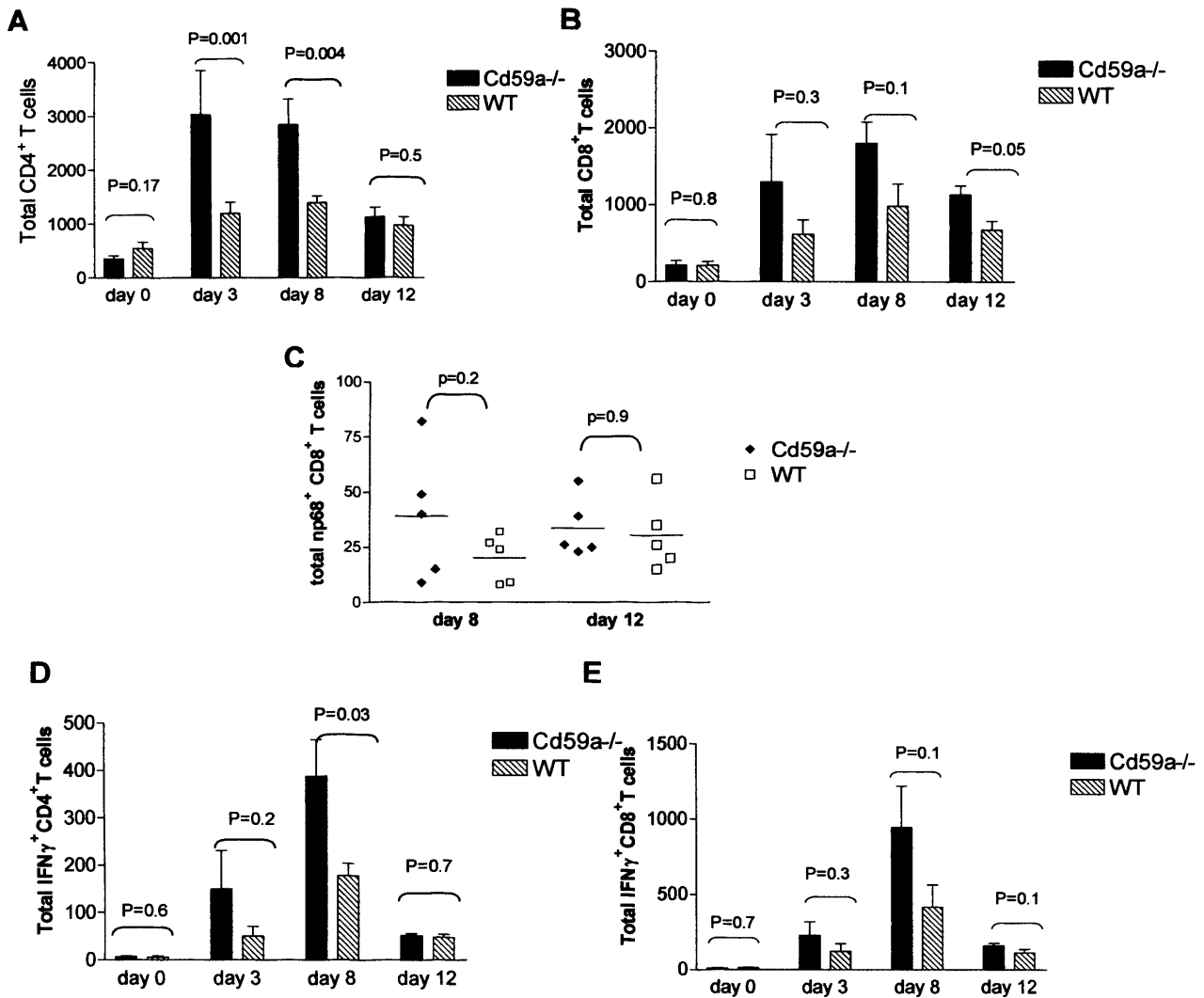
Figure 4.5. Cd59a-/- Mice Present Increased Numbers of Lung-infiltrated Lymphocytes after Influenza Infection

Cd59a-/- and WT mice (n=3/ group) were infected with 20HAU of influenza virus. Three, 8 and 12 days after infection, lungs were harvested and stained for CD4+ and CD8+ T cells. Naïve mice were used for day 0. Total numbers of CD4+ (A) and CD8+ (B) T cells were determined by flow cytometry. Total number of np68-specific CD8+ T cells was determined by tetramer staining (C). Lung homogenates were stimulated with PMA/ionomycin for 4 h and CD4+ (D) and CD8+ (E) IFN-γ producing T cells were determined by antibody intracellular staining. All results represent mean values ± SEM from at least 2 independent experiments.

4.2.4. Enhanced Influenza-specific CD4$^+$ T Cell Proliferation in Cd59a-/- Mice

To determine whether the increase in the number of CD4$^+$ T cells in the lungs of

Cd59a-/- mice was due to better priming of antigen-specific T cells, influenza-specific

CD4$^+$ T cell proliferation was examined. Cd59a-/- and WT mice were infected with

influenza virus and CD4$^+$ T cells were purified from spleens at day 8, 12 and 42 after

infection. Influenza-specific proliferation of CD4$^+$ T cells was increased in Cd59a-/-

mice compared to WT mice at all time points tested (Figure 4.6A and B). The

stimulation index (SI) was calculated by dividing the mean counts per minute (cpm) for

T cells plus antigen by the mean cpm for T cells alone. A SI of 2 or more was

considered positive. Cd59a-/- mice exhibited an SI > 3 for primary and memory

responses (Figure 4.6C and D respectively). In WT mice, a positive SI was observed

only at 12 days after infection (Figure 4.6C and D), indicating that influenza-specific T

cell proliferation is more readily observed in Cd59a-/- mice.

4.2.5. CD59 Expression Does Not Affect CD8$^+$ T Cell Cytotoxicity

As described above, total numbers of influenza-specific CD8$^+$ T cells recovered from

influenza-infected lungs were similar in Cd59a-/- mice and WT mice. IFN-γ

production by the CD8$^+$ T cells was also similar in both mouse groups. To confirm that

the CD8$^+$ T cell response to influenza was comparable in Cd59a -/- and WT mice, CTL

cultures were set up from infected mice at days 8, 12 and 42 post-infection and five

days later tested for killing of peptide-pulsed target cells. As expected, influenza virus

induced a strong CTL response and, as expected, the lytic activity of Cd59-/- mice was

comparable to WT mice (Figure 4.7).

4.2.6. Infection with Influenza Virus Induces Weight Loss in Balb/c.Cd59a-/- Mice

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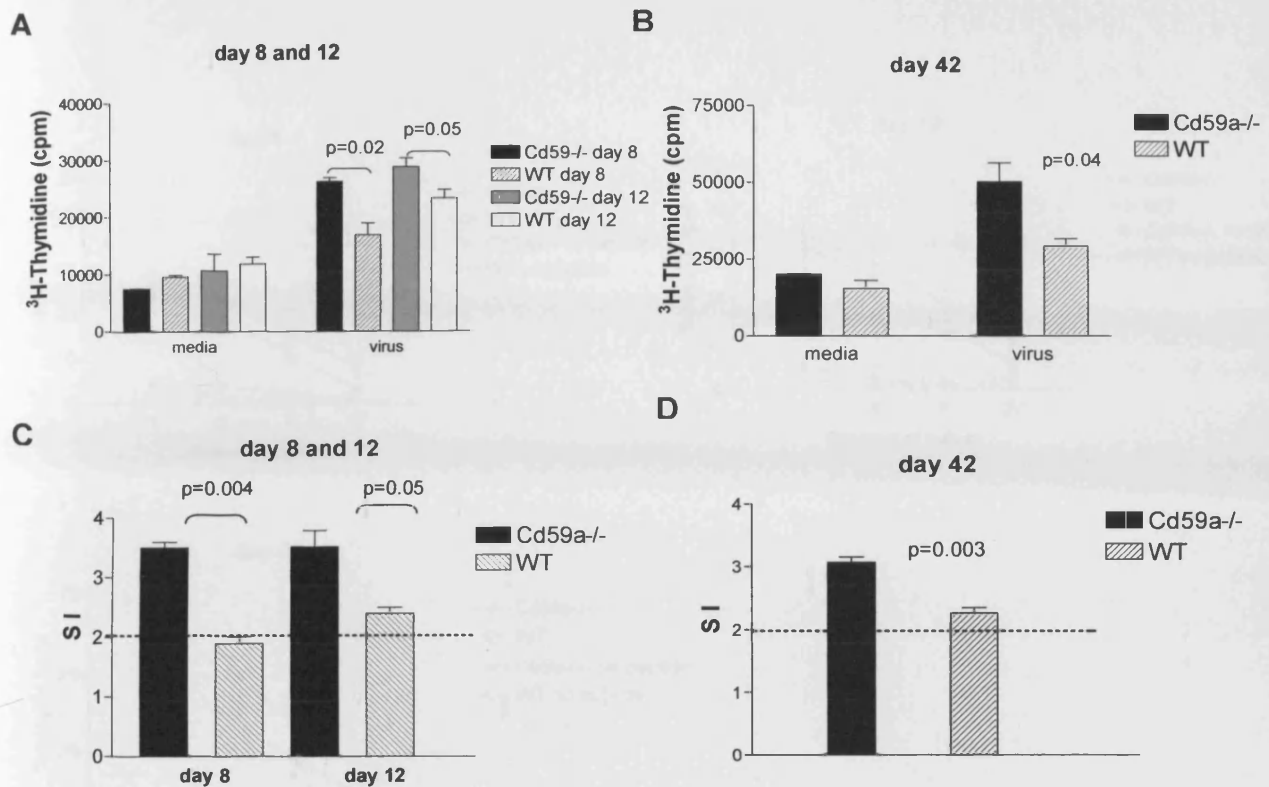
Figure 4.6. Enhanced Influenza-specific CD4^{+} T Cell Proliferation in Cd59a-/- Mice

Cd59a-/- and WT mice were infected with 20HAU of influenza virus. After 8, 12 (A) and 42 days (B), CD4^{+} T cells were purified from splenocytes and stimulated with APCs loaded with UV-inactivated virus. Influenza-specific proliferation was detected by ^{3}H-Thymidine incorporation at day 6. Stimulation index (SI) was calculated by dividing specific cpm by the background (C, D). Mice were analyzed individually and values shown are the means ± SEM (n= 3 mice/group). The results are representative of two independent experiments. Statistical significance was evaluated using Student's t test

day 8

% specific lysis

50
40
30
20
10
0

Dilution of cuture

1 3 9 27

—♦— Cd59a-/-
—□— WT
—●— Cd59a-/- no peptide
—○— WT no peptide

day 12

% specific lysis

50
40
30
20
10
0

Dilution of cuture

1 3 9 27

—♦— Cd59a-/-
—□— WT
—●— Cd59a-/- no pep
—○— WT no peptide

day 42

%specific lysis

75
50
25
0

Dilution of culture

1 3 9 27

—♦— Cd59a-/-
—□— WT
—●— Cd59a-/- no peptide
—○— WT no peptide
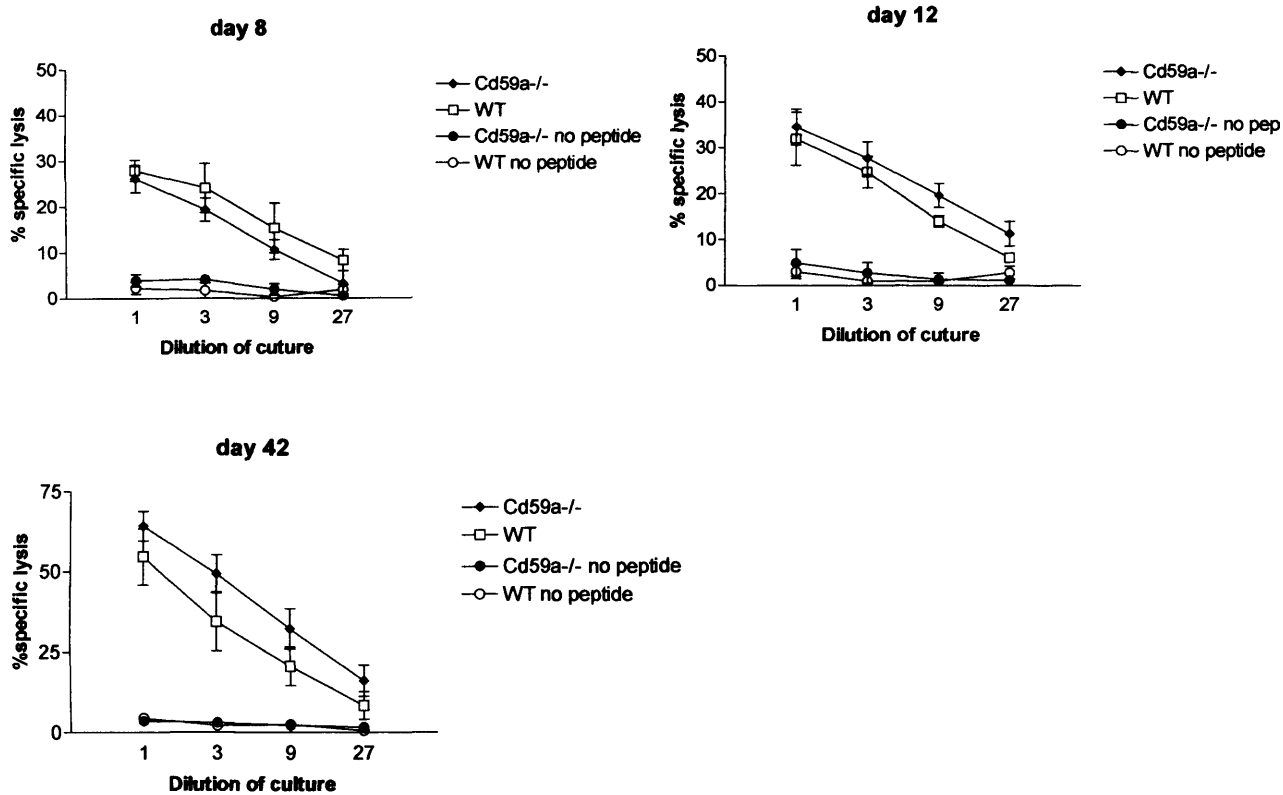
Figure 4.7. Influenza-specific CTLs in Cd59a-/- Mice

Cd59a-/- and WT mice were infected with 20HAU of influenza virus. After 8 (A), 12 (B) and 42 days (C) of influenza virus infection, CD8^{+} T cell cytotoxic activity was analyzed. Mice were analyzed individually and values shown are the mean ± SEM (n= 3 mice/group). The results are representative of two independent experiments. Statistical significance was evaluated using Student's t test (> 0.05 for all conditions tested)

Despite histological evidence of increased lung pathology in Cd59a-/- compared to WT mice, no clinical symptoms of influenza infection were observed in either group of mice. Indeed it has been reported that C57BL/6 mice such as those used in these experiments rarely display obvious signs of influenza-induced pathology such as weight loss whereas excessive Th1-type influenza-specific CD4$^+$ T cell responses in Balb/c mice correlate with measurable weight loss which is dose dependent and correlates with the extent of lung pathology (Hussell et al., 2001). Experiments were therefore performed to determine whether influenza-induced weight loss in Balb/c mice was exacerbated as a result of CD59a deficiency.

For this purpose, Balb/c mice lacking CD59a (Balb/c.CD59a -/-) were obtained. Prior to analysing the response of these mice to influenza virus infection, experiments were performed to confirm that the phenotype of Balb/c.CD59a -/- mice was similar to those on the B6 background. Specifically, the mice were tested to determine whether deletion of CD59 caused spontaneous vascular haemolysis and haemoglobinuria (Holt et al., 2001) as observed in B6 mice. Plasma and urine from Balb/c.Cd59a-/- mice contained increased amounts of haemoglobin when compared to Balb/c.WT mice indicating intravascular haemolysis and haemoglobinuria as reported previously for B6.Cd59a-/- mice (Figure 4.8).

Balb/c WT and Balb/c.Cd59a-/- mice were infected with a low dose of influenza virus and weight loss was monitored daily. Infection resulted in rapid weight loss in Balb/c.Cd59a-/- mice while body weight in Balb/c.WT mice remained almost constant (Figure 4.9). Since weight loss correlates with the extent of cellular
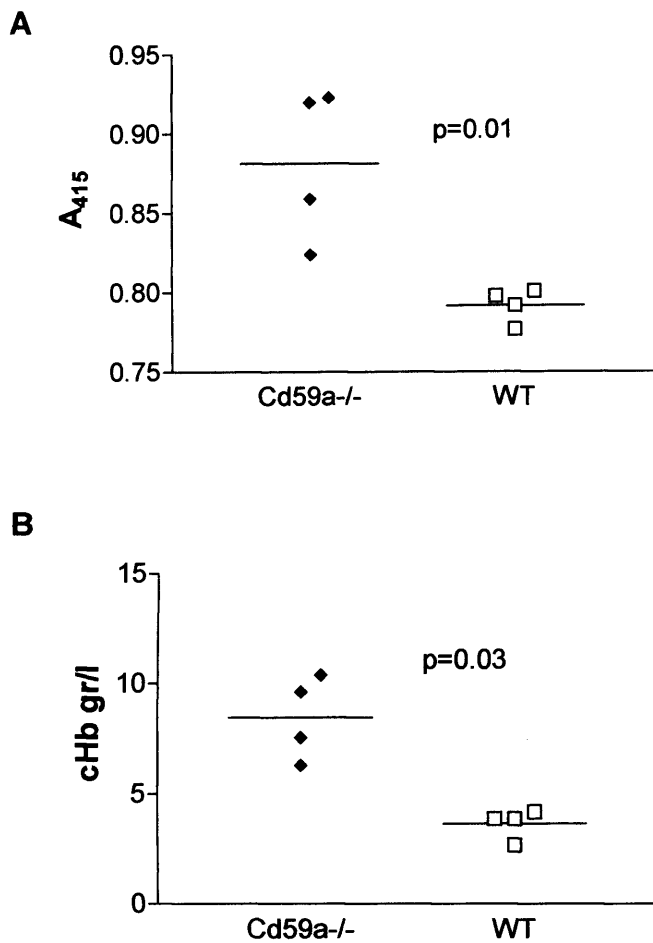
Figure 4.8. Intravascular Haemolysis and Haemoglobinuria in Cd59a-/- Mice
Urine from Balb/c.Cd59a-/- and Balb/c.WT mice was collected and haemoglobinuria
was measured by absorbance at 415 nm (A). Mice were bled into EDTA tubes and
haemoglobin concentration was measured in plasma as described in material and
methods (B). Each symbol represents an individual mouse. Statistical significance was
evaluated using Student's t test.
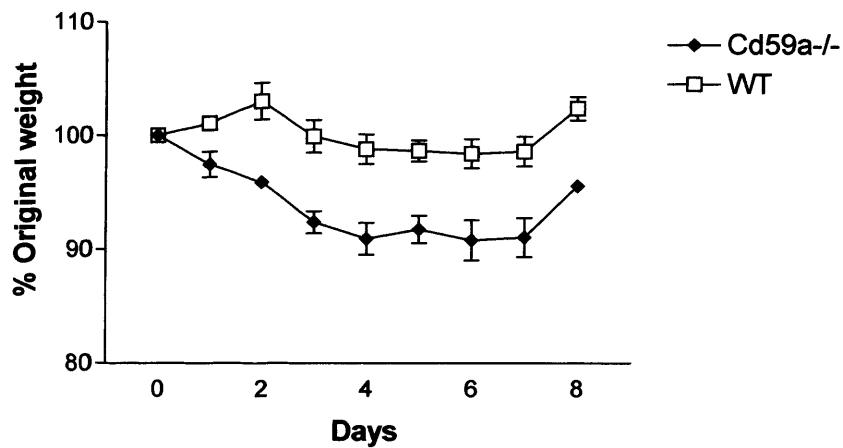
Figure 4.9. Influenza Infection Induces Weight Loss in Cd59a-/- Mice
Mice on the Balb/c background were infected with influenza virus and weight was monitored daily. Results represent mean values ± SEM of 5 mice per group. Statistic significance was evaluated by student's T test. P value was < 0.02 at all time points measured after infection.

infiltration into the infected lungs total numbers of macrophages, eosinophils and neutrophils were evaluated in both groups of mice by flow cytometry. Similar numbers of macrophages and eosinophils recruited to the lungs of both Balb/c.Cd59a-/- and Balb/c.WT (Figure 4.10A and B) however, numbers of neutrophils were elevated in Balb/c.Cd59a-/- mice at day 3 post infection (Figure 4.10C). T cell responses were also assessed. As expected, Balb/c.Cd59a-/- mice exhibited increased total numbers of lung-infiltrating CD4$^+$ T cells compared to WT mice (Figure 4.10D). Total numbers of infiltrating CD8$^+$ T cells were similar in both groups in line with the previous results obtained using B6 mice (Figure 4.10E). Overall, influenza infection of Balb/c.Cd59a-/- mice replicated the previous findings with B6.Cd59a-/- although total numbers of infiltrating cells in Balb/c.Cd59a-/- mice were greater than B6.Cd59a-/- mice, corresponding to the weight loss observed in the former group of mice.

4.2.7. Enhanced Cellular Infiltration in the Lungs of Cd59a-/- mice is Mediated by Complement Dependent and Independent Mechanisms.

In order to assess the effect of complement activation on lung infiltration in Cd59a -/- mice, complement was inhibit by daily injection of sCR1 (i.v.) for the first 3 days of influenza infection. After 3 days, lungs were harvested and total numbers of infiltrating cells were analysed by flow cytometry. No difference was observed in numbers of CD8$^+$ T cells, NK cells, macrophages and eosinophils after sCR1 administration (Figure 4.11A, B, C and D) although the increase in neutrophil number found in Cd59a-/- mice was normalised and comparable to WT mice after complement inhibition (Figure 4.11E). Similar to previous findings in mice infected with rVVG2 (Chapter 3), numbers of total infiltrating-CD4$^+$ T cell remained unchanged after sCR1
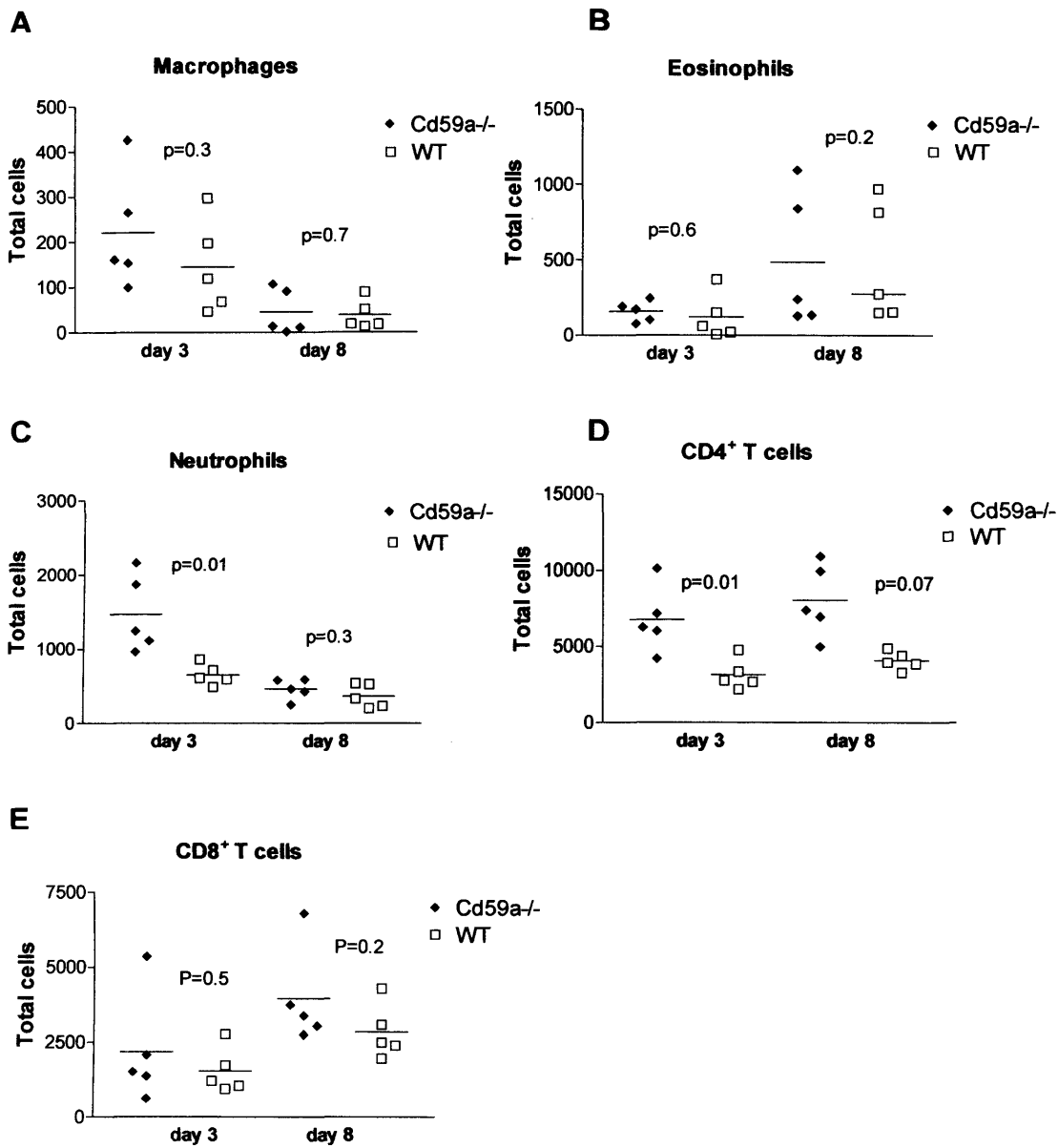
Figure 4.10. Enhanced Lung Inflammation in Balb/cCd59a-/- Mice
Cd59a-/- and WT mice (n=5/ group) on a Balb/c background were infected with
influenza virus. Three and 8 days after infection, lungs were harvested and infiltrating
cells were identified by flow cytometry. Total numbers of macrophages (A),
eosinophils (B) and neutrophils (C) were identified as described in Figure 4.3. Lung-
infiltrating cells were also stained for CD4$^+$ (D) and CD8$^+$ T (E). Each symbol
represents an individual mouse. Means are represented in all graphs. Statistical
significance was evaluated using Student's t test

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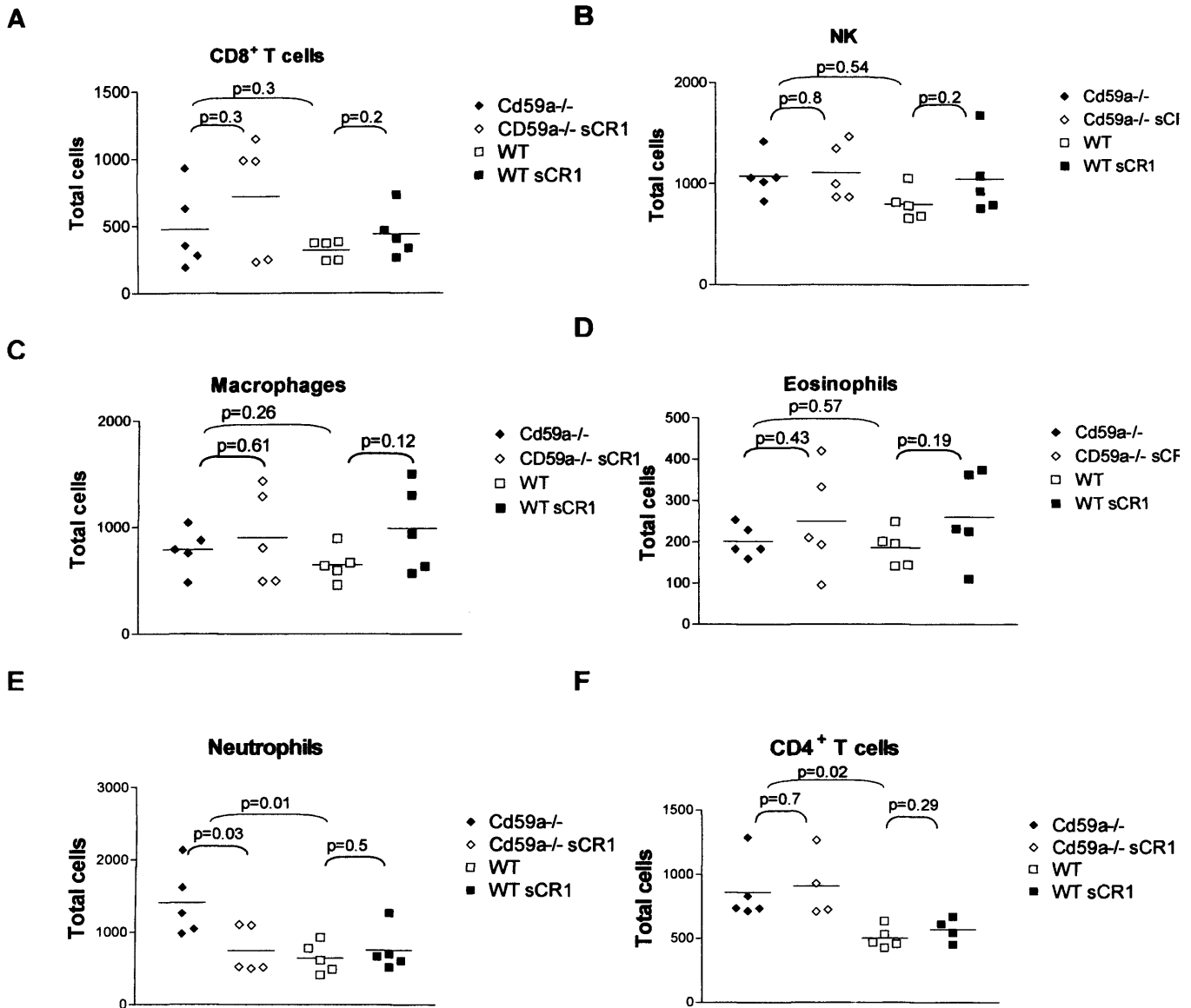
Figure 4.11. Enhanced Influenza-induced Lung Inflammation in Cd59a-/- mice is Mediated by Complement Dependent and Independent Mechanisms
Complement was inhibited *in vivo* by administration of sCR1 (i.v.) daily and mice were infected with influenza virus. After 3 days, lungs were harvested and total numbers lung-infiltrating cells were determined by flow cytometry. Total numbers CD8^{+} T cells (A) NK cells (B), macrophages (C), eosinophils (D), and neutrophils (E) were identified as described in Figure 3. Infiltrating-CD4^{+} T cells (F) were also identified by flow cytometry. Each symbol represents an individual mouse. Means are represented in all graphs. Statistical significance was evaluated using Student's t test.

administration, indicating that modulation of the CD4$^+$ T cell response by CD59a in experimental influenza infection is complement independent (Figure 4.11F). Overall, these data indicate that CD59a modulates the immune response to influenza virus by complement dependent (neutrophils) and independent (CD4$^+$ T cells) mechanisms.

4.2.8. CD4$^+$CD8$^+$ double positive cells in the lungs of infected mice

Phenotypic analyses of the cells infiltrating the lungs of influenza- infected mice revealed a novel population of CD4/CD8 double positive (DP) lymphocytes. These cells were analysed by two-colour flow cytometry at day 3 and 8 post-infection. At three days post-infection (Figure 4.12A), CD4/CD8 DP cells were observed in the lungs of both WT and Cd59a -/- mice but higher percentages were observed in Cd59a-/- mice. No DP cells were observed in either group by day 8 post-infection or naïve mice (Figure 4.12B). The percentages of the DP cells varied between 1% to almost 80 % in Cd59a-/- mice but did not exceed 40% in wt mice (Figure 4.13A). Surprisingly, CD4/CD8 DP cells were found only in the draining lymph nodes of Cd59a-/- mice (Figure 4.13B).

It is broadly accepted that the expression of CD4 and CD8 is mutually exclusive in mature lymphocytes. CD4/CD8 DP cells are present in the thymus where they differentiate into either CD4 or CD8 single-positive (SP) cells before emigrating into the periphery. Further characterisation of the cells by flow cytometry revealed that the DP cells were CD4^{intermediate} CD8^{high} and did not express CD11c, CD11b, NK1.1 or Gr1 (Figure 4.14). Expression of TCR and various T cell activation markers was then analyzed. The CD4/CD8 DP cells from isolated from both lungs and draining lymph-
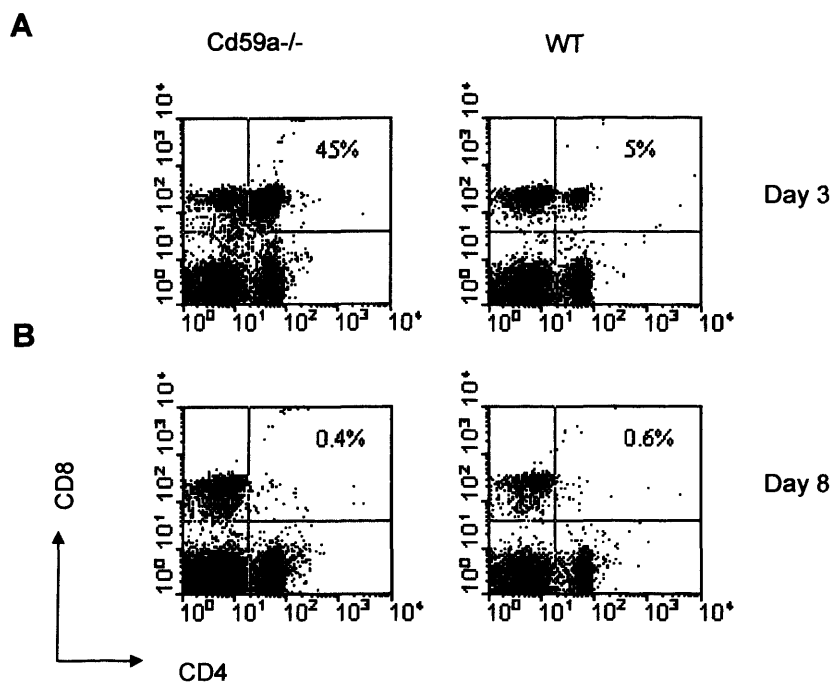
Figure 4.12. CD4/CD8 DP Cells in Lungs from Infected Mice
Mice were infected with influenza virus. At day 3 (A) and 8 (B) post infection, lungs
were harvested and infiltrating cells stained for CD4 and CD8. Representative Dot
plots of CD4/CD8 expression are shown.
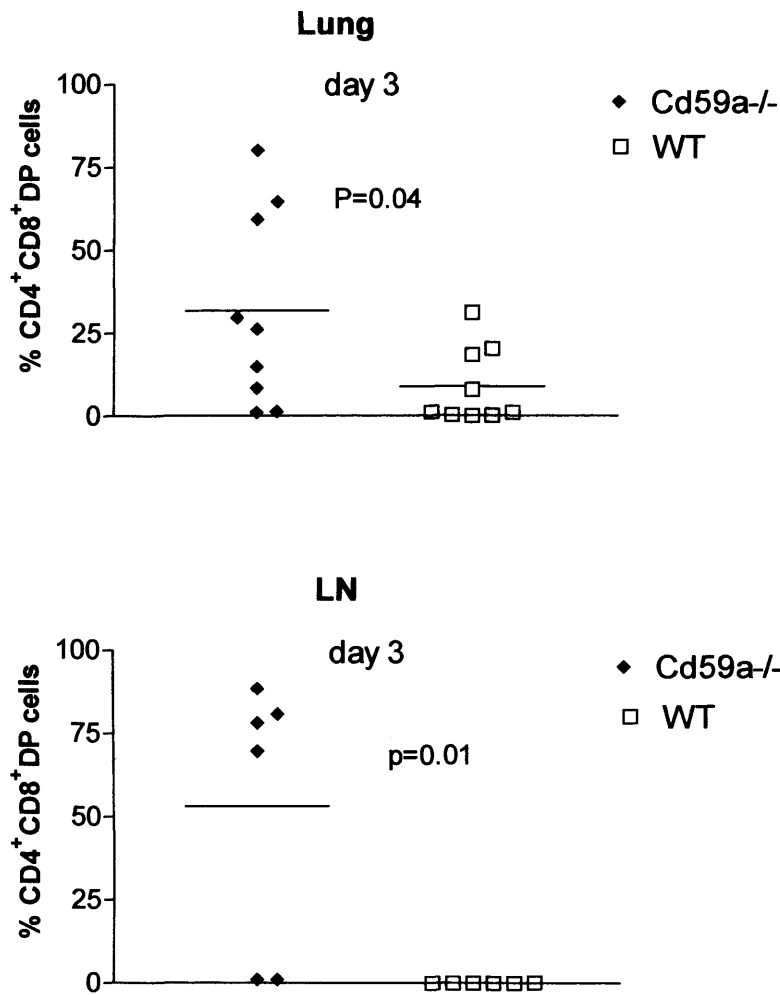
Figure 4.13. Increased Frequency of CD4$^+$CD8$^+$ DP Cells in Cd59a-/- Mice
Mice were infected with influenza virus. Three days post-infection, lungs and LN were harvested and analyzed for the expression of CD4 and CD8 by flow cytometry. Each symbol represents an individual mouse. Means are indicated in both graphs. Statistical significance was evaluated by Student's t test.
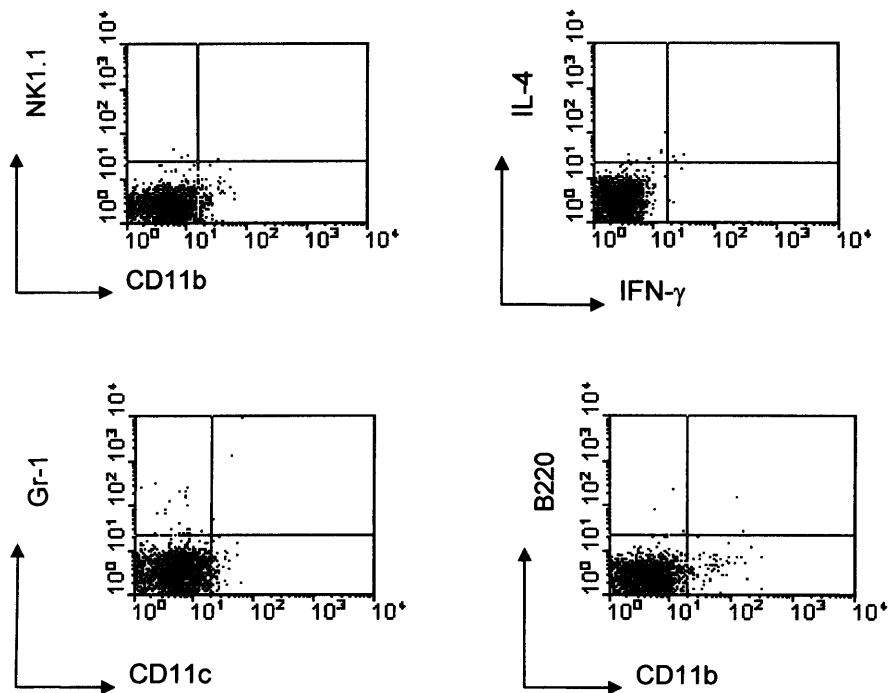
Figure 4.14. Phenotype Characterization of CD4/CD8 DP Cells

Gated CD4$^+$/CD8$^+$ DP cells from infected lungs were analyzed for the expression of NK1.1, CD11b, Gr-1, CD11c and B220. Lung homogenates were stimulated with PMA/ionomycin for 4 h and CD4$^+$/CD8$^+$ DP cells producing IFN-γ and IL-4 were determined by antibody intracellular staining

nodes of the infected mice did not express TCR, CD25, CD62L or CD44 (Figure 4.15).

DP cells have been found previously in mice infected with Chlamydia pneumoniae and

influenza virus. These cells, which were found in the lung within days of infection,

were CD4^{intermediate}CD8^{high} DP cells that did not express TCR and did not produce IFN-

γ upon stimulation (Pentilla *et al.*, 1998). Parallel staining of thymocytes showed a

similar phenotypic profile (Figure 4.15). This was an intriguing observation revealing

a possible thymic origin for CD4/CD8 DP cells.

To explore this possibility, mice were thymectomized, infected two weeks later

with influenza virus and lungs harvested 3 days post-infection. The experiment was

performed in Cd59a-/- mice due to the higher numbers and frequency of CD4/CD8 DP

cells in these mice compared to WT mice. Figure 16 shows the percentage of

CD4/CD8 DP cells infiltrating the lungs of thymectomized and control mice. Whilst

CD4/CD8 DP cells were found in 70% of control mice, DP cells were not found in any

of the thymectomized mice, indicating that the DP cells come from the thymus. The

functional significance, if any, of the cells remains unclear since the cells were unable

to produce IL-4 and IFN-γ after stimulation with PMA and ionomycin.
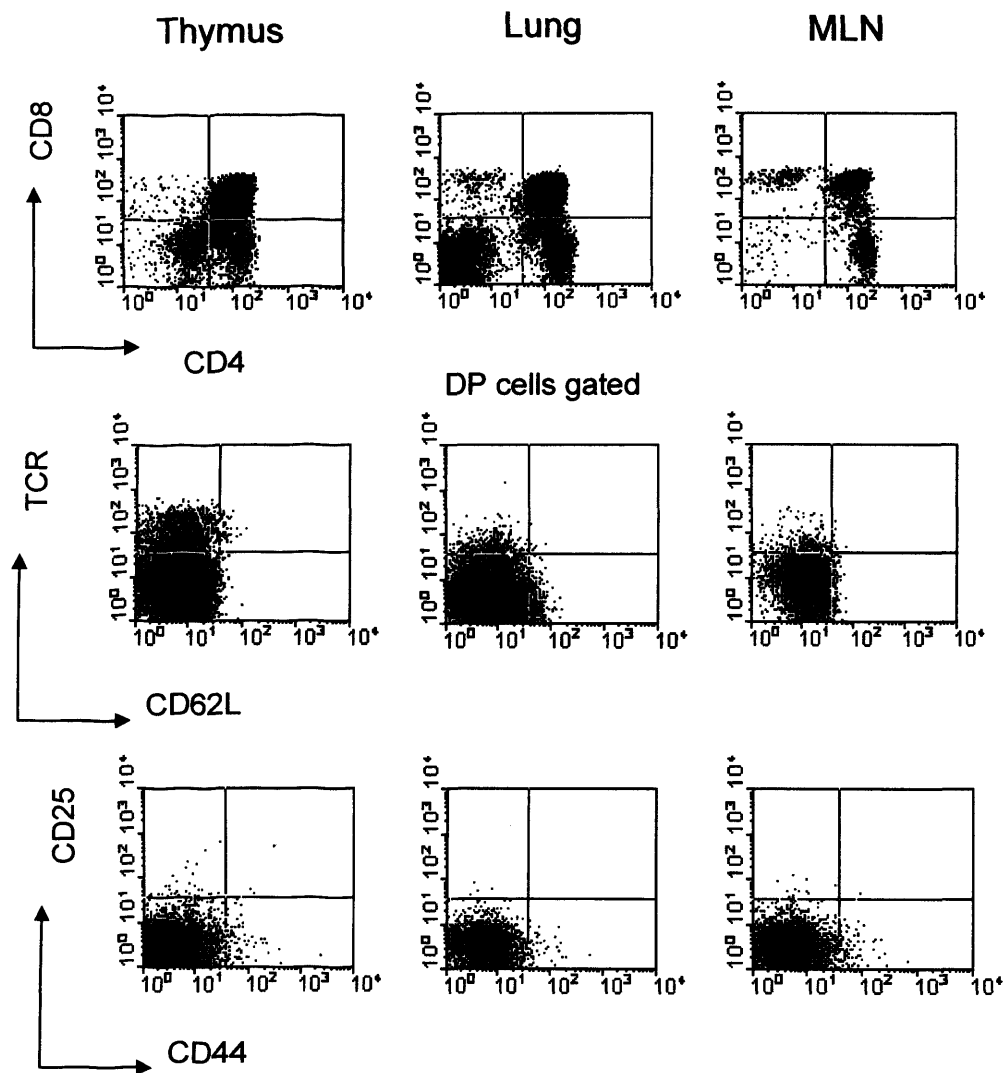
Figure 4.15. Characterization of CD4/CD8 DP Cells

Gated CD4/CD8 DP cells from infected lungs and draining LN were analyzed for the expression of T cell (TCR) and activation markers (CD25, CD44 and CD62L). Cells were stained in parallel with CD4/CD8 DP thymocytes.
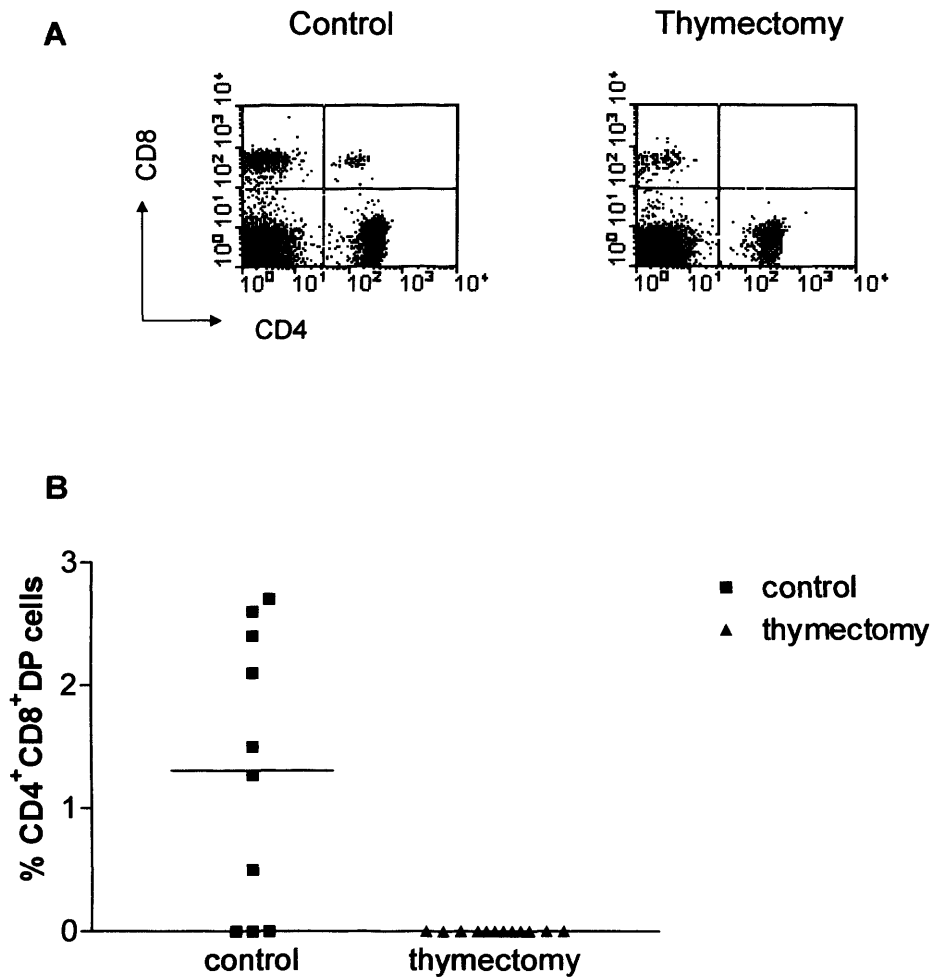
Figure 4.16. Origin of CD4/CD8 DP Cells

Cd59a-/- mice were thymectomized and, after two weeks, mice were infected with influenza virus. Presence of CD4/CD8 DP Cells was analyzed in lungs 3 days after infection. Non-thymectomized mice were used as controls. Representative dot plots of CD4/CD8 DP Cells in thymectomized and control mice are shown in (A). Percentages of CD4/CD8 DP Cells were calculated by flow cytometry (B). Each symbol represents an individual mouse and similar data was observed in two independent experiments. Mean are also indicated in each graph. Statistical significance was evaluated using Student's t test.

4.3. Discussion

The use of Influenza infection in mice provides a good model to study the immune

response in Cd59a-/- mice. Clearance of influenza virus is mediated by a concerted

activity of the innate immune system followed by antigen-specific T cells.

As discussed previously the complement system impacts on both innate and adaptive

immune responses to pathogens including influenza virus. The aim of the work

presented in this Chapter was to determine whether the complement regulator, CD59a,

plays an important role in limiting complement activation after influenza infection

thereby limiting the immunopathological consequences of infection with the virus.

Firstly, experiments were performed to determine whether the extent of

imunopathology was greater in the lungs of influenza-infected Cd59a -/- mice

compared to WT mice. Although lungs from infected mice were grossly normal in

terms of colour and size with only small red foci, a closer examination by histological

studies of H&E lung sections revealed increased lung injury in the Cd59a-/- mice

compared to WT mice. An important feature of Cd59a-/- infected lungs was the

prominent number of infiltrating cells. Characterization of the infiltrate revealed

greater numbers of neutrophils in Cd59a-/- than WT mice. Neutrophils are considered

a first line of defence against infection with pathogens. Their role has been well

investigated in terms of their importance in early protection against bacterial infection

(Mayer-Scholl *et al.*, 2004; Tatsukawa *et al.*, 1979; reviewed in Mayer-Scholl *et al.*,

2004; Segal, 2005). The relative contribution to virus clearance has not been intensely

investigated; however it has been suggested that neutrophils are important for

controlling virus replication in influenza-infected mice after antibody depletion

(Fujisawa, 2001; Ratcliffe *et al.*, 1988; Tumpey *et al.*, 1996; Tumpey *et al.*, 2005).

Simultaneously, they may also contribute to the overall pathogenesis by release of

immune mediators and subsequent T cell recruitment (Kobasa *et al.*, 2004; Mulligan *et*

al., 1993; Tumpey *et al.*, 2005). Accumulation of neutrophils in the lung was

dramatically reduced in sCR1-treated Cd59a-/- mice indicating that the enhanced

neutrophils response in the Cd59a -/- mice was due to enhanced complement activity

in these mice. In Cd59a-/- mice, more MAC formation occurs and, this increased MAC

deposition not may only generate more cell injury and lysis, but also serve to further

amplify complement activation and therefore the inflammatory response. Complement

fragments have been shown to induce neutrophils migration to lungs. Administration

of cobra vena factor (CVF) has been used to generate lung injury. In this model,

complement components generated as a consequence of systemic complement

activation by CVF, caused neutrophil migration and sequestration in lungs (Doyle *et*

al., 1997; Mulligan *et al.*, 1993). In addition, C3b deposition in HSV-infected

fibroblast enhanced neutrophil attachment to infected cells via receptor recognition

(Von Herrath *et al.*, 1996). As discussed in Chapter 1, the anaphylatoxins C3a and C5a

can act as chemoattractants of neutrophils (Martin *et al.*, 1997; reviewed in Guo and

Ward, 2005). However, CD59a regulates MAC assembly, and does not directly

influence production of C3a and C5a. It has been demonstrated that MAC formation

can indirectly influence the recruitment of inflammatory cells. For example, deposition

of sub-lytic amounts of MAC on human endothelial cells *in vitro* has been shown to

promote production of IL-8 and MCP-1, chemotactic for neutrophils and monocytes

respectively (Dobrina *et al.*, 2002; Kilgore *et al.*, 1997; Kilgore *et al.*, 1995).

Alternatively, absence of CD59a, by rendering cells more susceptible to MAC-

mediated damage or killing, might indirectly cause local complement activation and

generation of chemoattractants. Overall, the results described here imply that the absence of CD59a increases deposition of MAC that, directly or indirectly enhances neutrophil recruitment to the lung. In the absence of complement, neutrophil numbers in Cd59a-/- mice were comparable to WT mice. Inhibition of complement had no effect on neutrophil numbers in WT mice probably due to the efficient natural regulation of complement activation in these mice. Conversely, inhibition of complement had a significant effect on neutrophil numbers in Cd59a -/- mice because in the absence of CD59a, complement activation is more pronounced.

Influenza viruses exhibit inter-strain variation, differing markedly in their virulence and in their ability to cause lung pathology ranging from mild to severe. The severity of the lung pathology also appears to depend on genetic background of the host and can differ markedly amongst different mouse strains. In the majority of the experiments described here, B6 mice were infected with 20 HAU of the H17 virus. Using this dose of virus, influenza-induce immunopathology observed in those mice was mild with no weight loss and relatively little damage to the lung structure. Mice were also infected mice with more virulent virus strains (PR8 and X31) and higher doses (200 HAU per mouse. Maximal dose allowed) with no evident clinical manifestations. To further analyze the effect of CD59a deficiency on lung pathology WT and CD59a-deficient mice on the Balb/c background were analysed. Balb/c.Cd59a-/- mice showed a rapid decrease in body weight that remained low until day 8 post-infection. Body weight in Balb/c.WT mice was found to be constant through the duration of the experiment probably due to low dose of virus used. Increased numbers of neutrophils were also observed in lungs of Balb/c.Cd59a -/- mice compared to WT. Balb/c mice.

T cell responses were also analysed in WT and CD59a-deficient mice following infection with influenza virus. No difference was observed in the $CD8^+$ T cell response induced following infection of Cd59a -/- and WT mice with influenza virus, consistent with previous findings made using rVVG2 infected mice described in Chapter 3. Higher numbers of lung-infiltrating $CD4^+$ T cells were, however observed in influenza virus infected Cd59a-/- mice on both the B6 and Balb/c backgrounds. The kinetics of $CD4^+$T cell accumulation in lungs differed between Cd59a-/- mice and WT mice. The total number of $CD4^+$T cells on day 3 post-infection was dramatically increased in Cd59a-/- mice compared to WT mice and remained almost equally high at day 8 post infection. By day 12 post-infection, similar numbers were observed in Cd59a-/- and WT mice. The $CD4^+$ T cells from both groups of mice produced equal amounts of IFN-γ on a per cell basis but increased numbers of lung-infiltrated $CD4^+$ T cells in Cd59a-/- mice resulted in higher total levels of the cytokine, which is known to have direct anti-viral effects, at the site of virus replication. To determine whether increased $CD4^+$ T cell activity in Cd59a-/- mice facilitates more rapid virus clearance, virus titres were measured in the lungs by ELISA. Virus levels were undetectable at day 3 and 8 post-infection in Cd59a-/- and WT mice indicating that both groups of mice can efficiently control influenza virus infection (data not shown). In the previous chapter, rVVG2-specific $CD4^+$ T cell responses were enhanced in a complement independent manner in mice lacking CD59a. Since it has previously reported that complement activation promotes the activity of influenza-specific T cells in the lungs, it was possible that the effect of CD59a on T cell activity was an indirect effect attributable to complement (Baelder et al., 2005; Karp et al., 2001; Kopf et al., 2002). However, the results presented in this chapter demonstrate that inhibition of complement activity using sCR1 did not prevent the enhancement of $CD4^+$ T cell

responses observed in the Cd59a -/- mice, indicating that the effect of CD59a-deficiency on $CD4^+$ T cell activity is complement independent.

The analyses of cells infiltrating the lungs of influenza infected WT and Cd59a -/- mice revealed a population of CD4/CD8 DP cells in the lungs at day 3 but not day 8 post-infection. Higher percentages of these cells were seen in the lungs of Cd59a -/- mice compared to WT, and DP cells were found only in the draining lymph nodes of the Cd59a -/- mice. Phenotypic examination of the cells indicated that they were similar to DP cells isolated from thymus. Indeed, DP cells were not found in mice thymectomised prior to influenza infection. It has been reported that a high proportion of peripheral CD4/CD8 DP cells are present during the neonatal period in mice and newborn humans (Bonomo *et al.*, 1994; Griffiths-Chu *et al.*, 1984; Kotiranta-Ainamo *et al.*, 1999). In the mouse study, CD4/CD8 DP cells were detected in the lymph nodes but not spleens of 3- to 4-day-old mice, the percentage of which was found to vary greatly from mouse to mouse and which disappeared by 10 days of life (Bonomo *et al.*, 1994). The results of the study presented here imply that DP thymocytes can also escape from the thymus of adult mice, following infection. The mice used in this study were 4 – 6 weeks old, and it is possible that in older mice where thymic output is considerably reduced (usually at around six month of life), these cells would no longer be apparent (Scollay *et al.*, 1980). In adult rats, premature thymocytes can escape into the periphery as demonstrated by intrathymic injection of FITC and subsequent tracking of the FITC-labelled cells in the periphery (Hosseinzadeh and Goldschneider, 1993; Jimenez *et al.*, 2002). The increased incidence of DP cells in influenza-infected Cd59a-/- mice compared to WT mice may be a consequence of the more profound inflammatory response to influenza observed in these mice. The fate of the cells in the

influenza-infected mice described here is unclear. It is possible that the cells are not evident at day 8 post-infection due to cell death by apoptosis following export from the thymus. Intrathymic injection of FITC previous to influenza infection would allow the fate of the cells to be followed after export from the thymus into peripheral sites.

Several previous studies have also identified CD4/CD8 DP cells in the context of virus infection. In mice, presence of CD4/CD8 DP cell has been described after reovirus, adenovirus and influenza virus infection (Hillemeyer et al., 2002; Pentilla et al., 1998; Periwal and Cebra, 1999). CD4/CD8 DP cells have also been found in peripheral blood from healthy humans, although the frequency of the cells is reportedly increased during infections with e.g. human immunodeficiency virus (HIV) and Epstein-Barr virus (EBV) (Imlach et al., 2001; Ortolani et al., 1993; Tonutti et al., 1994; Weiss et al., 1998).The latter study demonstrated that circulating $CD4^{lo}CD8^{high}$ DP cells were mature cells that had lost CD1a-expression and were positive for the activation-memory marker CD45RO (Imlach et al., 2001; Nascimbeni et al., 2004; Ortolani et al., 1993). In hepatitis C virus (HCV)-infected patients, CD4/CD8 DP cells were shown to specifically respond to MHC class II- and MHC class I-restricted HCV antigens by IFN-γ production and were found in the liver, the site of infection (Nascimbeni et al., 2004). The authors of this study suggested that CD4/CD8 DP cells are fully activated T cells that have acquired the expression of the other co-receptor. Indeed, experiments performed in vitro have shown that expression of CD4 on $CD8^+$ T cells can be induced after stimulation with CD3- and CD28-specific antibodies, mitogens or DCs (Kitchen et al., 2004; Sullivan et al., 2001). Furthermore, purified $CD4^+$ T cells activated and cultured in the presence of IL-4 have been shown to

express the α-chain of CD8, an effect that is accompanied by increased cell proliferation and IFN-γ release by the cells (Molteni *et al.*, 2002; Paliard *et al.*, 1988). Recently Baba and colleagues identified a population of CD4/CD8 DP cells at sites of myosin-induced myocarditis, which were derived from monocyte-macrophage precursors and exhibited cytotoxic activity (Baba *et al.*, 2005). Overall, these studies, performed both in rodents and humans, indicate that CD4/CD8 DP cells with different phenotypic and functional characteristics have been identified implying that several different types of DP cells exist. Whether any of these cells play an important role within the immune system is yet to be established.

In summary the results of the experiments described in this Chapter indicate that influenza-induced immunopathology, defined by scoring lung pathology, is exacerbated in mice lacking CD59a. Increased pathology correlated with an increase in the number of lung-infiltrating neutrophils and CD4$^+$ T cells. Higher CD4$^+$T cell infiltration displayed in the Cd59a-/- mice was complement independent while accumulation of neutrophils in the lung was dramatically reduced in sCR1-treated Cd59a-/- mice implying a dependence on complement activation. These findings clearly demonstrate that CD59a impinges on the immune response to influenza by at least two mechanisms: complement-dependent (neutrophils) and independent (CD4$^+$T cells).

Chapter 5- Investigating Mechanisms of CD4$^+$ T Cell Modulation by CD59a

5.1. Introduction

As discussed in previous chapters, deficiency of CD59a significantly enhanced the CD4$^+$ T cell response to virus infection via a complement-independent mechanism raising the possibility that CD59a functions as a negative regulator of adaptive immunity *in vivo*.

There is clearly a need to regulate T cell responses *in vivo*. Clonal expansion of CD4$^+$ T cells is tightly regulated, as unchecked growth can result in expansion of autoreactive effector cells leading to autoimmune disease; or over-exuberant immune responses to foreign antigens leading to immunopathology. Several mechanisms are known to contribute to the regulation of T cell growth including activation-induced cell death (AICD), suppression by regulatory T cells and the delivery of negative signals via signalling molecules expressed on the cell surface (reviewed in Frauwirth and Thompson, 2002; Parijs and Abbas, 1998; Sakaguchi, 2004).

5.1.1. Activation Induced Cell Death (AICD)

Fas (CD95/APO-1) is a transmembrane receptor which belongs to the tumor necrosis factor receptor superfamily. Fas expression is upregulated on activated T cells and upon engagement with its ligand, FasL, mediates AICD, whereby the T cell dies by apoptosis (Nagata, 1997). Surface expression of Fas/APO-1 is known to play a critical role in regulation of CD4$^+$ T cell growth (reviewed in Parijs and Abbas, 1998) since

mice lacking expression of Fas, exhibit lymphoproliferative disease illustrating the importance of the Fas − FasL pathway in regulating homeostasis of naïve T cells and restricting the growth of activated T cells (Adachi *et al.*, 1995; Watanabe-Fukunaga *et al.*, 1992).

As mentioned in Chapter 1, patients with PNH exhibit clonal expansion of abnormal haematopoietic cells expressing a mutated form of the PIG-A gene that is involved in the biosynthesis of GPI-anchors. As a result, a proportion of blood cells from all lineages (myeloid, erythroid, mega-karyocytic and lymphoid) are deficient in surface expression of GPI-anchored proteins such as CD59 (Johnson and Hillmen, 2002; Parker, 1996).It has been observed that patient cells lacking CD59 proliferate more extensively than CD59^{+} cells from the same patient. The proliferative advantage of the CD59- cells has been attributed to the relatively low expression of Fas in the membrane of the CD59- cells compared to those that express CD59 (Brodsky *et al.*, 1997; Chen *et al.*, 2000). It is conceivable therefore that CD4^{+} T cells lacking CD59a also express lower levels of Fas, are therefore less susceptible to AICD and proliferate more extensively in response to antigen as described in Chapters 3 and 4.

5.1.2. Regulatory T Cells

There is accumulating evidence that T-cell-mediated regulation represents another mechanism for controlling T cell growth *in vivo*. It is now apparent that a population of CD4^{+} T cells called regulatory T cells (Treg) have the ability to control immune responses *in vivo* (Sakaguchi *et al.*, 1995). Based on intense research during the last decade, naturally occurring Treg cells (CD4^{+}CD25^{+} Treg) have emerged as an important T cell population for preserving peripheral tolerance to self-antigens.

CD4$^+$CD25$^+$ Treg develop in the thymus and are characterised by expression of CD25,

CTLA4, GITR or the transcription factor, Foxp3 (reviewed in Fehervari and

Sakaguchi, 2004; Jonuleit and Schmitt, 2003). The cells do not proliferate following

stimulation through their T cell receptor but can inhibit the proliferation of

conventional T cells (Sakaguchi *et al.*, 1995; Thornton and Shevach, 1998) and

reviewed in (reviewed in Sakaguchi, 2004). Tregs with the same phenotypic and

functional characteristics have been identified in mice and humans (reviewed in

Jonuleit and Schmitt, 2003). Although the cells have been studied mainly in the

context of their ability to inhibit the activity of autoreactive T cells, there is also a

strong body of evidence to support a role for naturally occurring Tregs in modulation

of immune responses to pathogens (Groux *et al.*, 1997; Takahashi *et al.*, 1998;

reviewed in Rouse and Suvas, 2004). As well as naturally occurring Tregs that arise in

the thymus, Tregs can also be induced in the periphery. These cells, which exhibit

phenotypic and functional characteristics that overlap with naturally occurring Tregs,

have been termed adaptive Tregs and also suppress the activity of conventional T cells

(reviewed in Roncarolo *et al.*, 2001; Weiner, 2001). The possibility that enhanced

CD4$^+$ T cell responses in Cd59a -/- mice are due to defective Treg activity was

therefore tested as described in this chapter.

5.1.3. Negative Cell Signalling

Engagement of the Ag receptor on T lymphocytes triggers a complex signalling

network that culminates in cell activation and differentiation. T cells require at least

two signals for full activation. One signal is delivered by the binding of the

antigen/MHC complex on the APC to an antigen-specific receptor on the T-cell

surface. The best characterized second or costimulatory signal is delivered by the

interaction between a cell-surface receptor on the T cell (CD28) with its ligands, CD80

(B7-1) and CD86 (B7-2) on the APC. When T cells are triggered through the TCR in

the absence of an appropriate costimulation signal, they may enter a quiescent state

known as anergy. Multiple costimulation pathways both positively (CD40/CD40L and

ICOS/ICOS-L) and negatively (B7.2 / CTLA4, PD-1/PD-1L) regulate T-cell function

(reviewed in Frauwirth and Thompson, 2002; Greenwald *et al.*, 2002). Thus T cell

activation is regulated by a balance of positive and negative signals mediated by a

series of costimulatory ligand–receptor pairs. In addition, T cell responses can be

modulated by inadequate cell priming by APCs. Ligation of ILT3 (Immunoglobulin-

like transcript 3) with an as yet unidentified ligand or the MyD-1 (CD172) receptor

with CD47 on APCs has been shown to inhibit APC activation affecting antigen

presentation and T cell priming (Brooke *et al.*, 1998; Cella *et al.*, 1997; Smith *et al.*,

2003) Whether CD59a delivers a negative signal to the T cell either directly or via the

APC will be explored in this chapter.

Overall therefore, the objectives of the work described in this Chapter were to

investigate 1) whether CD4$^+$ T cells from Cd59a -/- mice were less susceptible to

AICD, 2) whether suppression of T cell responses by Treg cells is defective in Cd59a -

/- mice and 3) whether CD59a delivers a negative signal to the T cells directly or

indirectly via effects on APCs.

5.2. Results

5.2.1. Susceptibility to Fas-mediated Apoptosis

As shown previously in Chapter 3 (Figure 3.6B), $CD4^+$ T cells from Cd59a-/- mice divided more extensively in response to stimulation with APCs and CD3-specific antibodies compared to WT mice. In order to determine whether this was due to less apoptosis in T cells isolated from the Cd59a -/- mice, three days after stimulation the cells were stained for annexin V expression. The percentage of proliferating cells that were annexin V positive was similar between the groups (Figure 5.1). Also, expression of Fas on $CD4^+$ T cells was equally up-regulated upon activation of $CD4^+$ T cells from Cd59a-/- and WT mice (Figure 3.11).

In order to confirm that $CD59a^+CD4^+$ T cells were not more susceptible to Fas-mediated killing than $CD59a^-CD4^+$ T cells, purified $CD4^+$ T cells from Cd59a-/- and WT mice were activated with CD3- and CD28-specific antibodies and cultured for 24 hours with soluble FasL (sFasL) containing a Flag tag plus Flag-specific antibodies necessary to cross-link sFasL for induction of Fas-mediated killing. Addition of 1/50 dilution of sFasL induced apoptosis in the majority of $CD4^+$ T cells as measured by annexin V and PI staining and both groups of cells displayed a similar response to increasing concentrations of cross-linked sFasL (Figure 5.2A).

Results obtained in Chapter 3 showed that modulation of $CD4^+$ T cell activation by CD59a requires the presence of APCs. Susceptibility of the $CD4^+$ T cells to Fas-mediated apoptosis was therefore investigated in the presence of APCs. Total splenocytes were activated with CD3-specific antibodies and apoptosis was induced with cross-linked sFasL. The level of apoptosis induced by sFasL was comparable in

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Figure 5.1. CD59-CD4$^+$ T Cells Are not More Resistant to Apoptosis
Purified CD4$^+$T cells were activated with CD3-specific antibodies in the presence of APCs. After 3 days, proliferated cells were stained for annexin and analyzed by flow cytometry. Histograms are representative of two independent experiments.

CD59a⁻ and CD59⁺ CD4⁺ T cells (Figure 5.2 B and C). Together this data indicates that CD59a-expressing T cells are not more susceptible to Fas-mediated apoptosis.

5.2.2. An Evaluation of CD4$^+$CD25$^+$ Regulatory T Cells in Cd59a-/- Mice.

As discussed previously, Treg play an important role in controlling immune responses. The most striking property of CD4$^+$CD25$^+$ Treg cells is their ability to suppress the proliferative responses of CD4$^+$CD25$^-$ T cells (Takahashi *et al.*, 1998; Thornton and Shevach, 1998; Thornton and Shevach, 2000). A relative defect, in terms of number or function, of these Tregs could be responsible for CD4$^+$ hyper-responses in Cd59a-/-. To test this hypothesis, CD4$^+$CD25$^+$ T cells from naïve Cd59a-/- and WT mice were enumerated. As shown previously in Figure 3.11 (Chapter 3) (day 0), no significant difference in the percentage of CD4$^+$CD25$^+$ T cells from naïve Cd59a-/- and WT mice was found. Next, the suppressive capacity of CD4$^+$CD25$^+$ Treg was tested *in vitro*. When co-cultured with CD4$^+$CD25$^+$ Tregs, proliferation of CD4$^+$CD25$^-$ T cells upon incubation with CD3-specific antibodies and APCs was reduced in a dose dependent manner (Figure 5.3). However, no significant difference was observed in the capacity of CD4$^+$CD25$^+$ Tregs isolated from WT and Cd59a -/- mice to mediate suppression. Furthermore, CD59a-/- or WT responder cells were equally susceptible to the suppressive effects of CD4$^+$CD25$^+$ Tregs (Figure 5.3). These findings indicate that CD4$^+$CD25$^+$ Tregs in CD59a-/- mice are similar in number and function to those in WT mice.

T cells, whose immunosuppressive activity is induced/acquired in the periphery, have also been identified. Inducible Treg subsets include Tr1 and Th3 cells, whose major effector molecules are the immunosuppressive cytokines IL-10 and TGF-ß,

A

B

Cd59a-/-   WT

Control
Anti-Flag

9.3%   3.7%   4.3%   2.4%
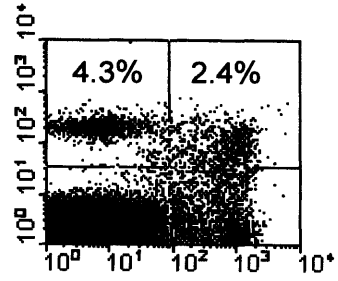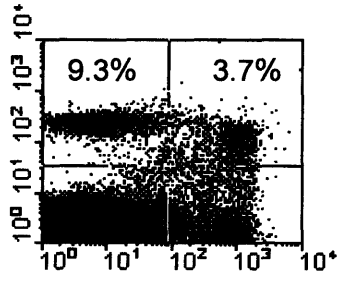
1/100 sFasL
+ anti-Flag

6.9%   6.3%   3.5%   3.3%
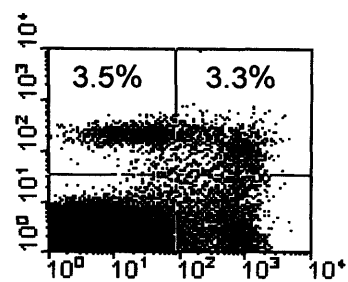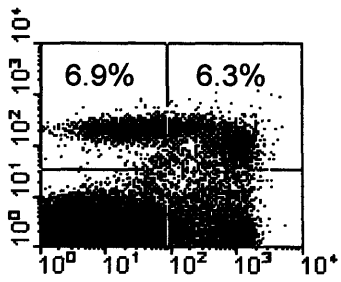
1/50 sFasL
+ anti-Flag

5.9%   7.6%   2.5%   4.1%

CD4

Annexin
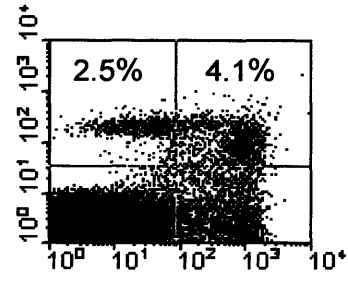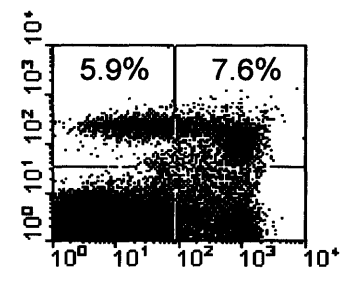
Gated on CD4$^+$ T cells
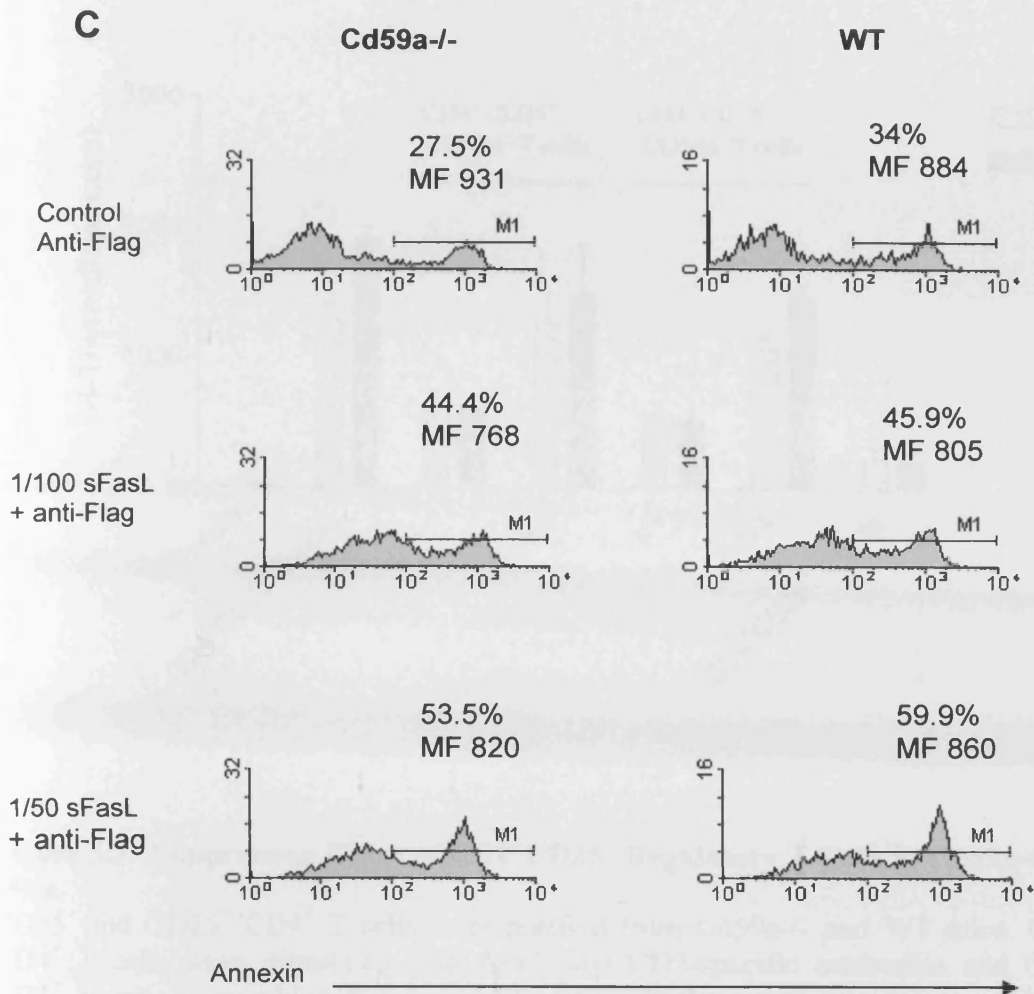
Figure 5.2. Induction of Apoptosis on CD4$^+$ T Cells

Cells were incubated 24 h with sFasL and cross-linked with Flag-specific antibodies to induce apoptosis. Purified CD4$^+$T cells were incubated with CD3- and CD28-specific antibodies for 24 h. Apoptosis was detected by annexin and PI specific staining (A). Total splenocytes were stimulated with CD3-specific antibodies. Apoptosis was analyzed by annexin staining in total cells (B) or in the CD4+-gated population (C). Histograms and dot-plots shown are representative of two independent experiments.
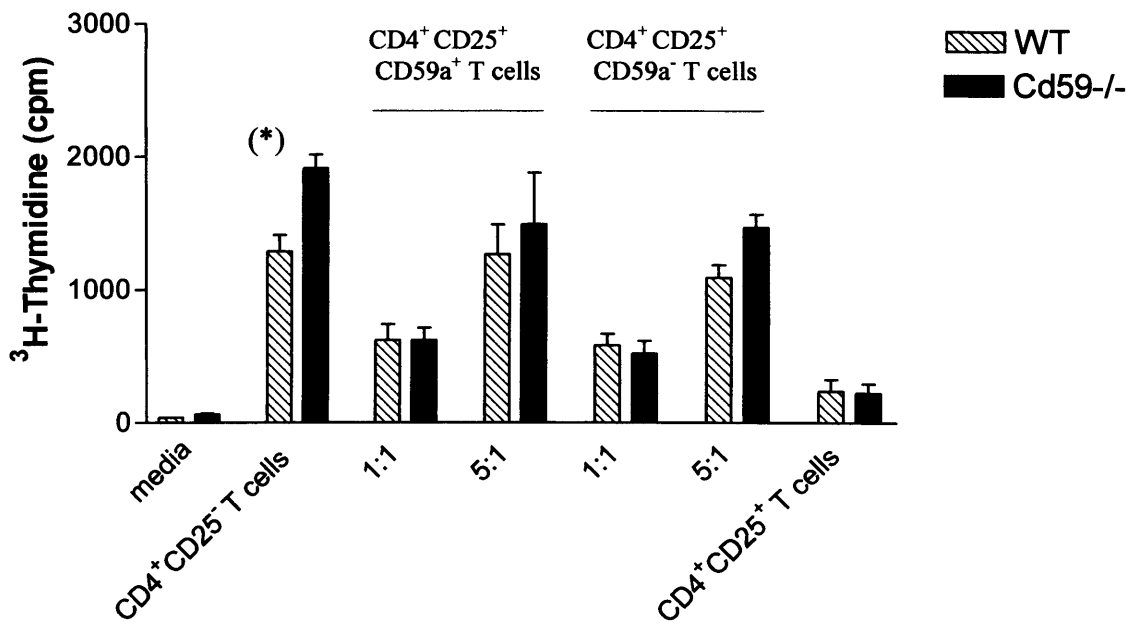
**Figure 5.3. Suppression Effect of CD4⁺CD25⁺ Regulatory T Cells in Cd59a-/-
Mice.**

CD25⁻ and CD25⁺ CD4⁺ T cells were purified from Cd59a-/- and WT mice. CD25⁻
CD4⁺ T cells were stimulated with APCs and CD3-specific antibodies and CD25⁺
CD4⁺ T cells were added at 1:1 and 5:1 ratios. Proliferation was measured by ³H-
Thymidine incorporation. Values shown are the mean ± SD Statistical significance (*)
was evaluated using Student's T test (p<0.01).

respectively (reviewed in Fehervari and Sakaguchi, 2004; Steinman *et al.*, 2003). It is possible that there is a defect in the generation of these cells in Cd59a -/- mice. This hypothesis is based on the idea that Treg cells are induced in WT mice following infection with influenza virus that serve to limit the expansion of the virus-specific T cell response. Thus, in the case of Cd59a-/- mice, an inability to generate these Treg cells would result in greater expansion of influenza-specific $CD4^+$ T cells as described in Chapter 4. If this is true, the expansion of influenza-specific $CD59a^-CD4^+$ T cells should be limited in the presence of influenza-primed $CD4^+$ T cells from WT mice. An experiment was therefore performed whereby purified $CD4^+$ T cells from Cd59a-/- and WT mice were labelled with the fluorescent, membrane-intercalating dyes PKH26 (red) or PKH67 (green) respectively and cotransferred into T cell deficient Rag-/- mice. One day after cell transfer, mice were either left uninfected or challenged with influenza virus and three days later, the mice were sacrificed and lungs were evaluated for the presence of fluorescent cells by flow cytometry. No infiltrating cells were observed in the lungs of uninfected mice however, an increased percentage of $CD59a^-$ $CD4^+$T cells was found (2 fold increase, Figure 5.4) indicating that, under similar conditions of cell priming, $CD4^+$ T cells from Cd59a-/- mice showed enhanced cell proliferation that was not due to impaired generation of inducible Tregs. If that had been the case, Tregs generated from WT $CD4^+$ T cells should have equally suppressed both cell types.

5.2.3. CD59a ligand on APCs

Cd59a-/- mice, as discussed in previous chapters, exhibited enhanced $CD4^+$ T cell responses *in vivo* and *in vitro* compared to $CD4^+$ T cell from WT mice. This effect was shown to be complement-independent and required the presence of APCs implying
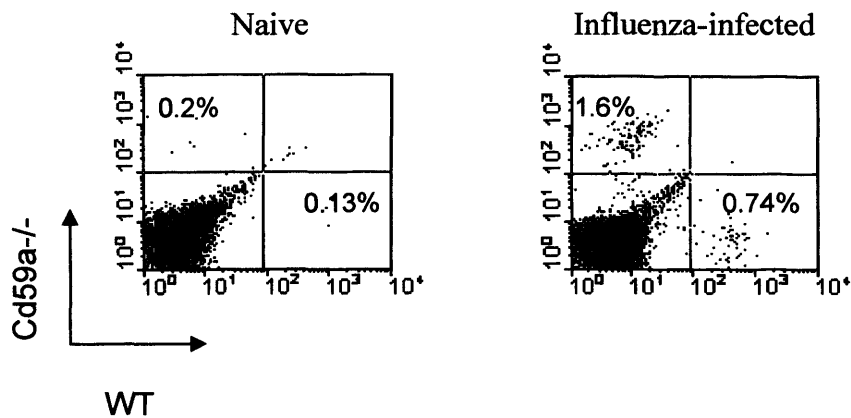
Figure 5.4. Co-transfer of Fluorescently Labelled CD59a⁺ and CD59a⁻ CD4⁺T Cells.

CD59a⁻ (PKH26, red) and CD59a⁺ (PKH67, green) labelled T cells from naïve mice, were transferred to recipient mice by intravenous injection. One day later, the mice were challenged with Influenza virus. After an additional 3 days, mice were sacrificed, and fluorescence cells were identified by flow cytometry. Representative dot plot of two independent experiments are shown.

that CD59a on T cells interacts with a ligand, other than a complement component, on APCs. To test this hypothesis a fluorescently labelled CD59a-IgG$_{2a}$ fusion protein (made in the laboratory by Dr B. Sivasankar) was used to examine whether all or a subset of murine splenocytes bound CD59a. The CD59a-IgG$_{2a}$ fusion protein did not bind neutrophils, NK cells, CD8$^+$ and CD4$^+$ T cells but did bind to macrophages, DCs and B cells (Figure 5.5). Interestingly therefore, it appears that a ligand for CD59a is selectively expressed on cells attributed with antigen presentation capacity.

5.2.4. Effect of CD59a ligation on APC

CD59a is anchored to the cellular membrane through a GPI linkage and, due to the lack of a cytoplasmic domain, is unable to activate signalling pathways directly. The data presented in previous chapters seems to indicate the existence of a ligand for CD59a on APCs, which may be signalling competent. The possibility that engagement of CD59a does not modulate the CD4$^+$ T cell response directly, but rather indirectly through CD59a-ligand signalling on APCs was therefore considered (Figure 5.6). In order to test this hypothesis, proliferation of CD4$^+$ T cell from Cd59a-/- mice was assessed in the presence of irradiated CD59a$^+$CD4$^+$ T cells. The reasoning behind this experiment was that CD59a expressed on irradiated WT CD4$^+$ T cells would engage with its ligand on the APC and, if this interaction resulted in delivery of a negative signal to the APCs, this would prevent the enhanced proliferation normally observed for CD4$^+$ T cells purified from Cd59a -/- mice. Proliferation of CD59a$^-$CD4$^+$ T cells was not affected by the presence of irradiated CD59a$^+$CD4$^+$T cells suggesting that engagement of CD59a does not affect APC function (Figure 5.7).

To further explore the effect of CD59a engagement on APC function, effects of CD59a engagement on DC maturation and antigen presentation were analysed. DCs
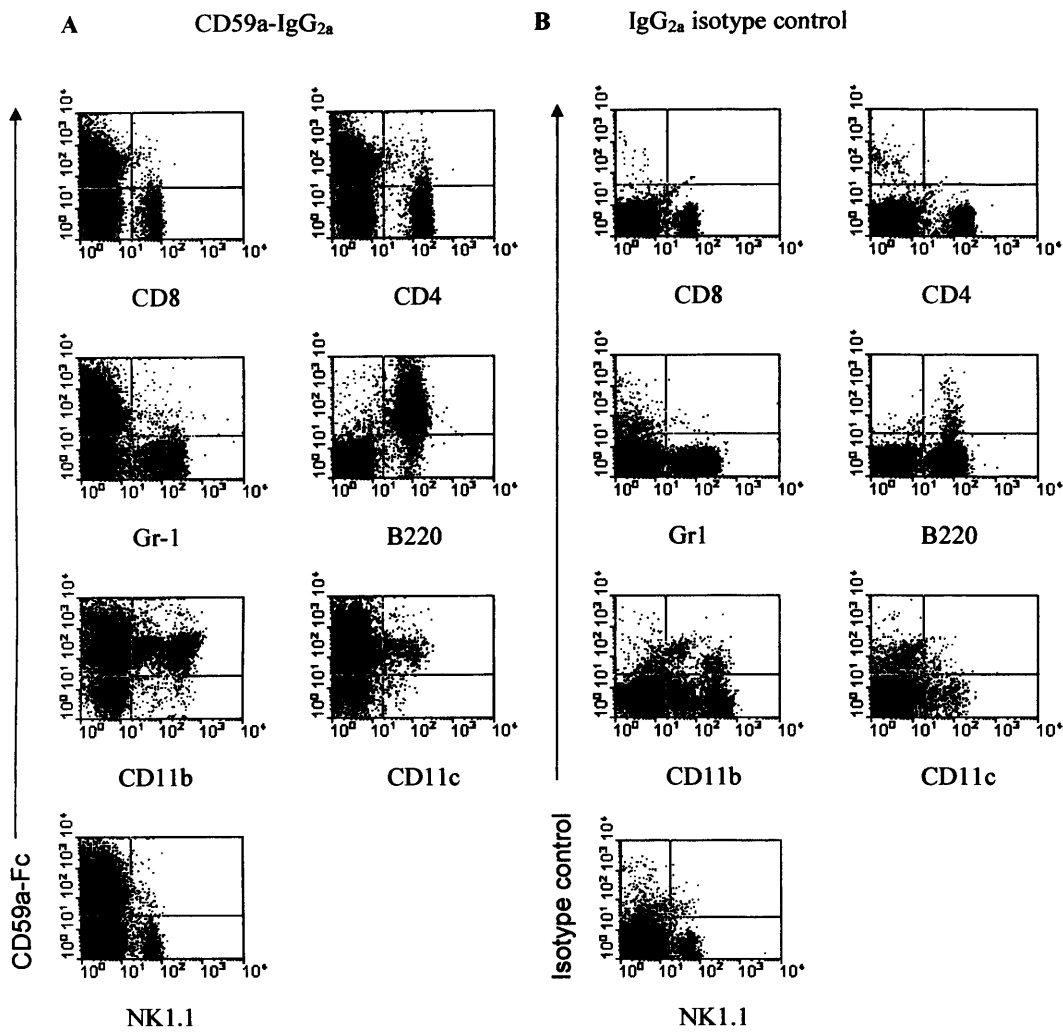
Figure 5.5. CD59a-Fc fusion Protein Binds to APCs
Splenocytes were double staining with biotinilated- CD59a-IgG$_{2a}$ fusion protein (y
axis) and antibodies to CD4, CD8, Gr-1 (granulocytes), B220 (B cells), CD11b (
Macrophages), CD11c (DCs) and NK1.1 (NK cells) (x axis) (A). As a control for non-
specific staining, cells were staining with a biotynilated-mouse IgG2a antibody
(MEM-43).Stains are representative of two independent experiments.
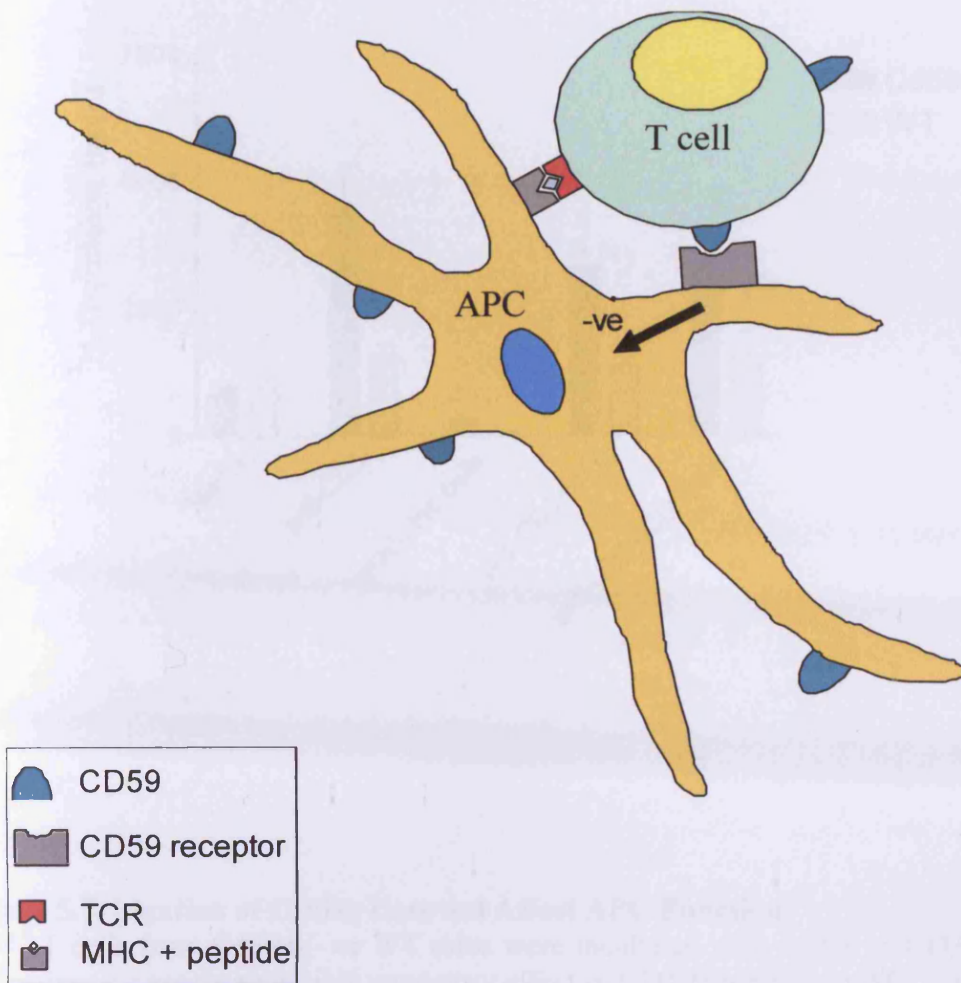
Figure 5.6. CD59 Delivers a Negative Signal to the APC

Direct interaction of CD59 on the T cell and a specific ligand on the APC results in an inhibitory signal being transmitted to the APC resulting in down modulation of APC activity and consequently, T cell.
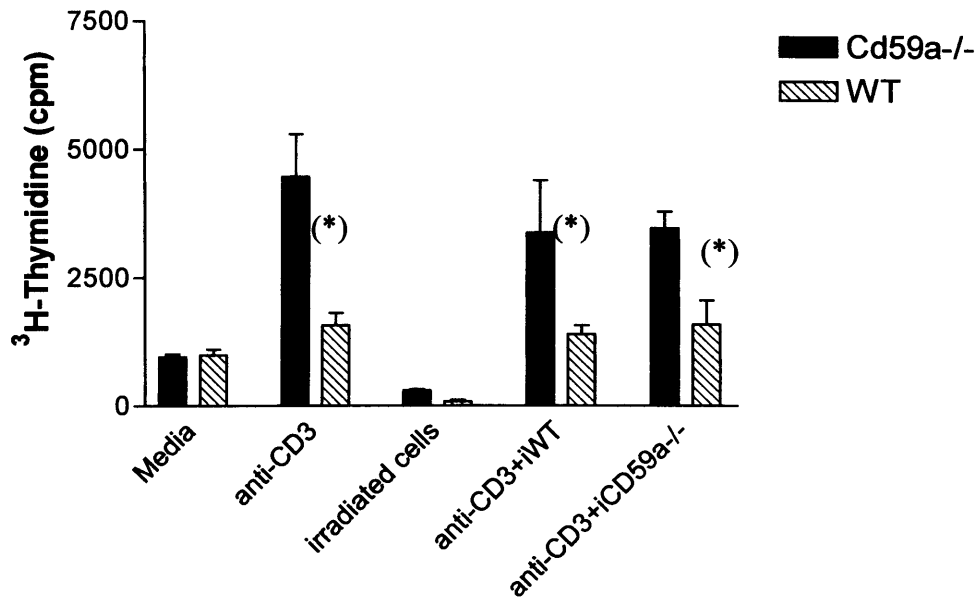
Figure 5.7. Ligation of CD59a Does not Affect APC Function.
CD4$^+$ T cells from Cd59a-/- or WT mice were incubated with APCs or CD3-specific antibodies. To assess a possible regulatory effect of CD59a binding to APCs, irradiated CD4$^+$ T cells from both groups of mice were added to the well. Proliferation was measured by ^{3}H-Thymidine incorporation. Values shown are the mean ± SD and statistical significance (*) was evaluated using Student's T test (p<0.05). Data is representative of two independent experiments.

are the most potent APCs and possess the ability to stimulate naïve T cells. Immature

DCs (iCDs) are characterized by high endocytic activity and low T-cell activation

potential and, when stimulated, DCs differentiate into mature DCs (mDCs)

upregulating MHC class II and co-receptors such as CD86, greatly enhancing their

ability to activate T-cells (McIntire and Hunt, 2005). Infection with influenza virus has

been shown to mature DCs (Cella *et al.*, 1999). In order to test if ligation of CD59a

ligand impinges on DCs maturation, immature DCs were incubated with $CD4^+$ T cells

from Cd59a-/- and WT mice and influenza virus when applicable. DC maturation was

assessed by up-regulation of MHC class II and CD86. No difference was observed on

maturation of DCs recovered after CD59a-/- or WT $CD4^+$ T cell-DC co-culture (Figure

5.8). The antigen presenting capacity of these cells were then tested in an influenza-

specific proliferation assay. DCs, previously incubated with either CD59a -/- or WT T

cells and influenza virus, were used to prime influenza specific-$CD4^+$ T cell purified

from an infected WT mouse. As shown in figure 5.9, no difference was observed in T

cell priming by DCs cocultured with either CD59a-/- or WT $CD4^+$ T cells, indicating

that expression of CD59a on $CD4^+$ T cells does not alter the antigen presentation

capacity of DCs (Figure 5.9).

	CD86	**MHCII**
DCs	27% MF 127 _{M1}	25.8% MF 130 _{M1}

	CD86	**MHCII**
DCs	27% MF 127 M1	25.8% MF 130 M1
DCs+ Flu	46% MF 99 M1	66% MF 175 M1
DCs+ Flu + CD4+CD59a- T cells	71% MF 106 M1	77.8% MF 191 M1
DCs+ Flu + CD4+CD59a+ T cells	68.5% MF 103 M1	79% MF 182 M1
DCs+ CD4+CD59a- T cells	31.6% MF 119 M1	25.4% MF 107 M1
DCs+ CD4+CD59a+ T cells	38.8% MF 122 M1	35% MF 113 M1
DCs+ LPS	94% MF 151 M1	96% MF 254 M1

Figure 5.8. CD59a Binding Does not Affect DCs Maturation

Immature BM-derived DCs were infected with 0.01 HAU/cell for 24 h to induce maturation. To study the effect of CD59a binding on DCs, purified naïve CD4+ T cells from Cd59a-/- or WT mice were added to DCs during maturation stimulus (ratio 3:1). DCs maturation was analyzed by up-regulation of CD86 and MHC-II expression. Representative histograms from two independent experiments are shown.
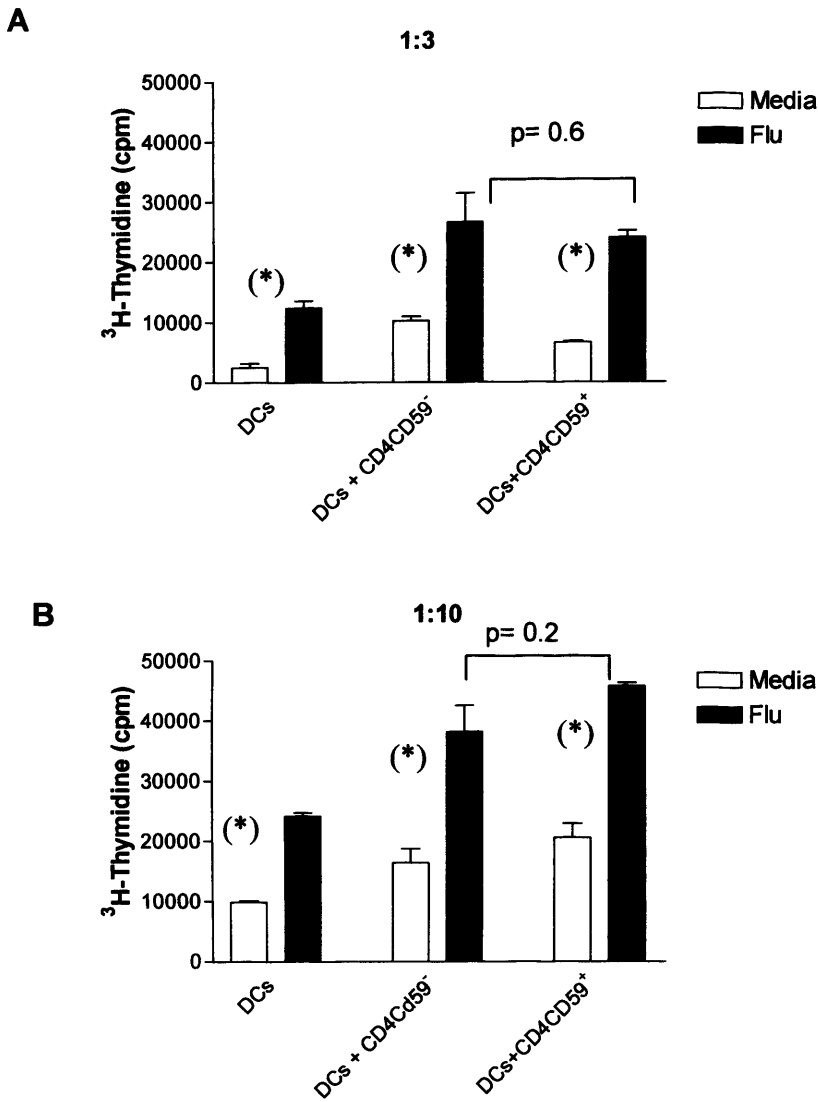
Figure 5.9. CD59a Binding Does not Affect Antigen Presentation on DCs

DCs previously incubated with Influenza virus and CD4+ T cells from Cd59a-/- or WT mice, were used as APCs for influenza-specific CD4+ T cells proliferation. WT mice were infected with 20 HAU of influenza virus. After 12 days, splenocytes CD4+ T cells were purified and used as responders. 10^5 responder cells were incubated with 3×10^4 (A) or 10^4 (B)DCs for 6 days. Uninfected-DCs were used as negative control. Proliferation was measured by ^{3}H-Thymidyne incorporation. Values shown are the mean ± SD and statistical significance (*) was evaluated using Student's T test (p<0.05). Data is representative of two independent experiments.

5.3. Discussion

The experiments described in this Chapter aimed to investigate the potential

mechanisms through which CD59a modulates CD4$^+$ T cell activity *in vitro* and *in vivo*.

The possibility that CD59a -/- T cells were less susceptible to AICD was investigated

since previous reports indicate an anti-apoptotic role for human CD59. Co-ligation of

both CD59 and Fas in Jurkat cells using cross linking antibodies dramatically

decreased cell death mediated by Fas while co-ligation of both CD28 and Fas

dramatically increased Fas-mediated apoptosis (Legembre *et al.*, 2006). The authors of

the study attributed the finding to CD59-mediated translocation of Fas into a specific

lipid microdomain causing modulation of its signalling pathway. Whether this

represents a physiological pathway through which CD59 and Fas interact is not

known. Other reports have indicated that clones from PNH patients, sorted based on

CD59 expression, exhibit reduced Fas expression and are therefore more resistant to

apoptosis. Whilst this finding implies a link between CD59 expression and resistance

to apoptosis, those cells lacking CD59 lack expression of all GPI-anchored proteins

therefore the effect on apoptosis is not necessarily due to lack of CD59 (Horikawa *et

al.*, 1997; Ware *et al.*, 1998). The findings of the experiments presented in this Chapter

do not support a role for CD59a in modulating the sensitivity of mouse CD4$^+$ T cells to

FasL-induced apopotosis.

Next, experiments were performed to determine whether regulatory T cell activity was

impaired in Cd59a -/- mice. Similar numbers and suppressive activity of CD25$^+$CD4$^+$

Tregs was found in Cd59a-/- mice compared to WT ruling out the hypothesis that the

level or activity of naturally occurring Tregs is impaired in the Cd59a -/- mice. The

possibility that Tregs are differentially induced in response to antigen stimulation in

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WT and Cd59a -/- mice was also investigated. A recent study showed engagement of the human CReg, CD46, on human CD4$^+$ T cells induced a regulatory T cell phenotype in the cells. In these experiments, co-engagement of CD46 and CD3, in the presence of IL-2, induced an IL-10–secreting Treg cell phenotype characteristic of Tr1 cells (Kemper *et al.*, 2003). Tr1 cells differentiate in the periphery from naive precursors, typically in the presence of interleukin-10 (IL-10), and regulate T-cell responses through their ability to produce IL-10 and TGF-β. The hyperactivity of CD59a-deficient T cells cannot however be attributed to a defect in generation of Tr1 cells since the hyperactvitiy was still evident after mixing the cells with WT T cells. Co-transfer of CD4$^+$ T cells from Cd59a-/- and WT mice also showed that the difference in cell proliferation was intrinsic to the CD59a-deficient CD4$^+$ T cell. However, the nature of this experiment does not rule out a difference in cell migration of CD4$^+$ T cells from Cd59a-/- and WT mice. In response to influenza-infection, CD59-CD4$^+$T cells may express integrins or chemokine receptors conferring increased migratory capacity to the lung.

5.3.2. Negative Cell Signalling

Indirect Effects via APCs

Signalling by GPI-anchored proteins is intriguing, because these proteins have no transmembrane or cytoplasmic domains. Therefore, it is possible that CD59a binding initiates intracellular signalling that is transduced through the CD59 ligand. Several receptors have recently been shown to modulate the activity of myeloid cells. Ligation of the CD200R with a recombinant protein and specific antibodies inhibited IFN-γ-induced TNF-α and IL-6 secretion by macrophages and was also shown to induce production of IL-10 and TGF-β by DCs resulting in generation of Tregs (Gorczynski *et*

al., 2005; Jenmalm *et al.*, 2006). The expression of the ligand CD200 was shown to be

upregulated on T cells upon activation (reviewed in Barclay *et al.*, 2002). Similar

effects were reported as a result of CD47-CD172 interacting on APCs and T cells.

Engagement of CD47 on DCs results in impaired maturation of the DCs and decreased

production of inflammatory cytokines (Demeure *et al.*, 2000), ultimately affecting

their ability to stimulate T cells. No such effect was observed for CD59a expressing T

cells since in the presence of T cell expressing CD59a, no defect in antigen

presentation or maturation was observed.

Direct Effects on T Cells

It is possible that engagement of CD59a on T cells downmodulates the signal delivered

to the T cells upon activation through the TCR (Figure 5.10). Antibody cross-linking

of CD59 on human T cells induces protein phosphorylation and, in

immunoprecipitation studies, CD59 was found to be associated with src kinases. This

finding implies that CD59 modulates T cell activation by associating with signalling

molecules found in the cell membrane. Indeed, CD59 is a GPI-anchored protein found

in lipid rafts and may therefore modulate T cell signalling by associating with other

proteins also found in lipid rafts. Translocation of signalling molecules in and out of

lipid rafts can control the ability of cells to respond to various stimuli. For example,

CD28 engagement has been shown to induce redistribution and polarization of GPI

microdomains at the site of TCR engagement and increases the presence of the linker

of activated T cells (LAT) in membrane rafts while CTLA-4 ligation effectively blocks

this increase and reduces raft expression (Margarita Martin 2001)
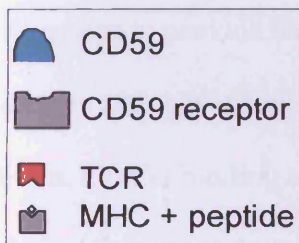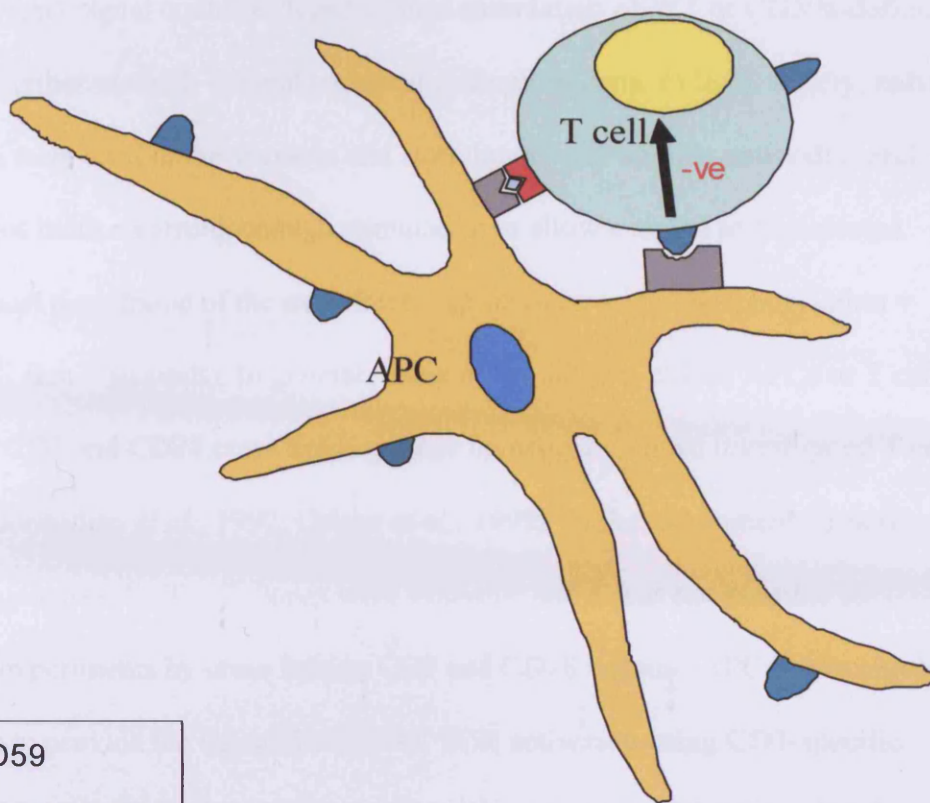
Figure 5.10. CD59 Negative Modulates Signal to the T Cell
Direct interaction of CD59 on the T cell and a specific ligand on the APC results in an inhibitory signal being transmitted to the T cell reducing the strength of the positive signal delivered through the TCR.
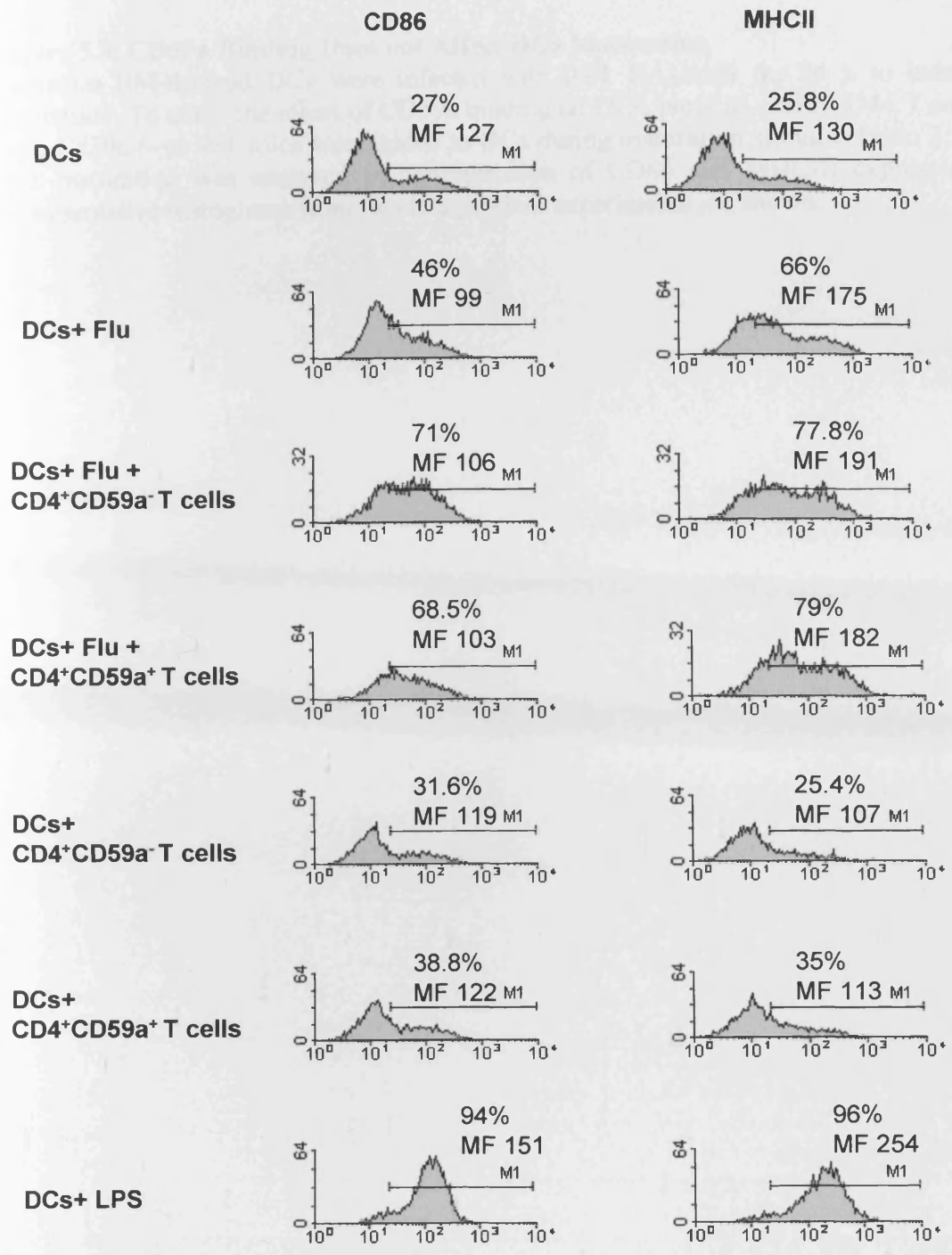
In an attempt to test the effect of CD59a on T cell activation, analyses of Ca^{2+} flux and protein tyrosine phosphorylation were performed on WT T cells and T cells lacking CD59a upon stimulation with CD3-specific antibodies and APCs. Unexpectedly, no signal could be detected after stimulation of WT or CD59a-deficient T cells using either method. Several technical difficulties came to light. Firstly, naïve lymphocytes were used in these assays and stimulation with soluble antibodies and APCs may not induce a strong enough stimulation to allow a signal to be detected within the short time-frame of the experiments (protein tyrosine phosphorylation = minutes, Ca^{2+} flux = seconds). In general, using either antigen-pulsed APCs or T cell clones or by CD3 and CD28 cross linking, other investigators have investigated T cell signalling (Donnadieu *et al.*, 1992; Geiger *et al.*, 1999). In the experiments described here, no antigen-specific T cell clones were available and it was not possible to perform the experiments by cross linking CD3 and CD28 because APCs were required in the assays to provide the ligand for CD59a. TCR activation using CD3-specific antibodies and APCs may not provide a sufficiently strong signal for detection. In addition, CD59a binding to its ligand might not occur simultaneously posing another limitation for assays described here. Additional work will therefore be required to explore in more detail the effect of CD59a on T cell activation. It may be possible to improve the experiments by back-crossing Cd59a-/- mice with $CD4^+$ TCR transgenic mice (eg. OTII, ovalbumin-specific) which will provide a source of antigen-specific $CD4^+CD59^-$ T cells that can be easily activated with peptide-loaded APCs. In this context, CD59a engagement and TCR activation will occur simultaneously and potentially solve the problems described above. Another possibility may involve activation of the T cells with beads coated with CD3-specific antibodies and the

CD59a ligand. Identification of the ligand for CD59a will be required for this approach to succeed.

In summary, the results of this Chapter rule out the possibility that CD59a modulates T cell activity through affecting Fas-FasL interactions, through regulatory cell activity or through delivery of a negative signal to APCs. Unfortunately due to the technical limitations discussed above, it has not yet proven possible to determine whether CD59a affects the strength of a signal delivered to a T cell upon TCR engagement. As mentioned above, it may be possible to address this issue in more detail using experimental systems whereby better strategies for detecting signalling events within T cells can be employed.

Chapter 6- CD59 Modulates Human CD4$^+$ T Cell Proliferation

6.1. Introduction

CD59 is highly expressed on human T cells and, although several studies imply that CD59 is involved in T cell activation, its specific function has not been well defined. Studies have shown that antibody cross-linking of CD59 induces a series of events including the activation of protein-tyrosine kinases, Ca^{2+} flux, IL-2 release and cell proliferation (Korty *et al.*, 1991; Stefanova, 1991). These events are not unique to CD59, since many other GPI-anchored proteins induce a series of similar events when cross-linked with specific antibodies (Gunter *et al.*, 1984; Kroczek *et al.*, 1986; Reiser, 1990; Thompson *et al.*, 1989). Controversy exists regarding the physiological relevance of these experiments. Since the GPI anchor proteins only transverses the outer leaflet of the membrane bilayer, it is likely T cell activation by cross-linking antibodies is the result of protein aggregation and association of CD59 with signalling molecules.

Studies *in vivo* with CD59a deficient mice, showed contradictory results. While antibody cross-linking of CD59 induced T cell activation, mice deficient in CD59a exhibited CD4$^+$ T cell hyperactivity as discussed in Chapter 3 and 4. The same findings were made in studies of other GPI-anchored proteins like CD55 and Ly6A (Heeger *et al.*, 2005; Liu *et al.*, 2005; Stanford *et al.*, 1997).

The purpose of this study was to investigate the effect of CD59 on human T cell activation. The aim was to conduct these experiments by blocking CD59 and measuring the effect of loss of CD59 function rather than to perform antibody cross-linking experiments as described above. The first part of this Chapter describes production of Fab fragments of CD59-specific antibodies and the latter part describes the impact of antibody binding on the activity of human T cells.

6.2. Results

6.2.1. Differential Expression of CD59 on Human CD4$^+$ T Cell Subsets

CD59 is widely distributed on most tissues and circulating cells including lymphocytes (Meri and Lachmann, 1991). In order to determine whether CD59 expression differs amongst different populations of CD4$^+$ T lymphocytes, CD4$^+$ T cells from healthy donors were purified using magnetic beads and phenotypically characterized using antibodies specific for T cell activation and memory markers.

Two isoforms of CD45 have been described that are differentially expressed on T cells upon stimulation (Clement *et al.*, 1988). While the low molecular weight isoform CD45RO is expressed primarily on memory T cells, CD45RA is expressed on cells with a resting/naïve phenotype (Akbar *et al.*, 1988). All CD4$^+$ T cells expressed high levels of CD59 (Figure 6.1A) but increased expression of CD59 was detected on CD4$^+$CD45RO$^+$ T cells compared to CD4$^+$CD45RA$^+$ T cells (Figure 6.1A and B). These data indicate that recently or previously activated T cells up-regulate expression of CD59. To further investigate this possibility, CD4$^+$ T cells were activated with CD3-specific antibodies and APCs, and expression of CD59 was compared in unactivated and activated cells. Upon stimulation, expression of CD59 was up-regulated on CD4$^+$ T cells as measured by an increase in mean fluorescence (Figure 6.2A). Moreover, acquisition of the activation marker CD25 by activated cells was accompanied by up-regulation of CD59 expression (Figure 6.2B). Thus, CD4$^+$ T cells appear to up-regulate CD59 expression upon activation but whether CD59 is involved in the process of T cell activation or whether it is upregulated simply to protect activated cells from complement attack remains to be clarified.
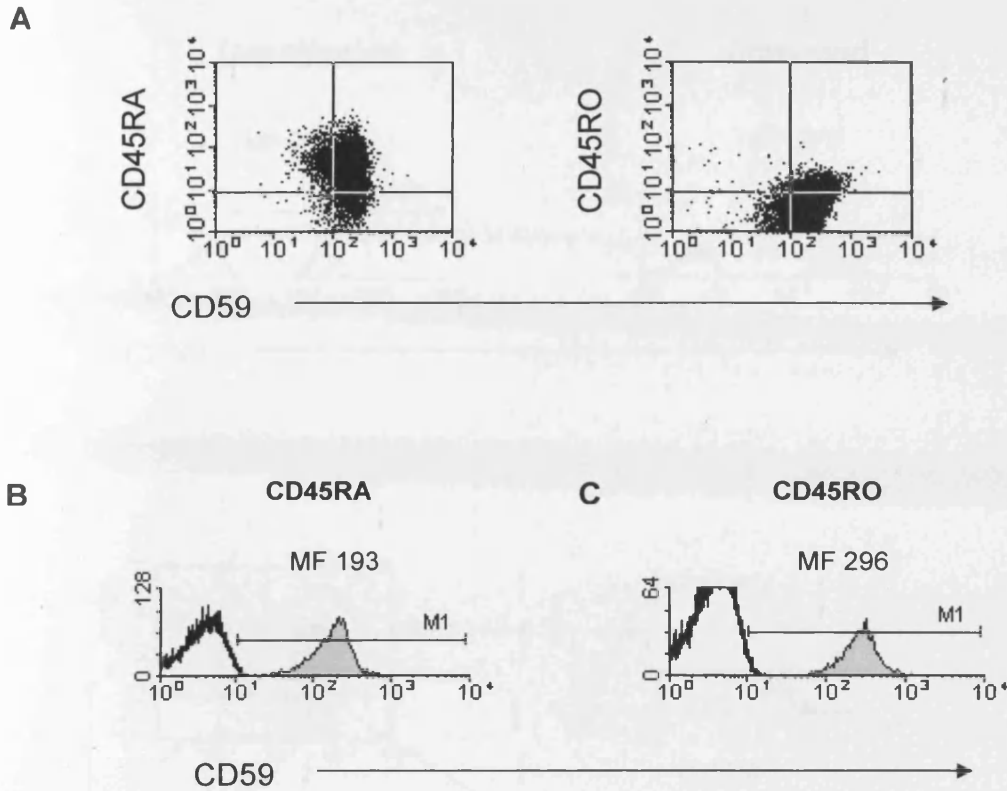
Figure 6.1. Expression of CD59 on Human CD4$^+$ T Cells

CD4$^+$ T cells were purified from human PBMCs. Cells were stained with CD59-, CD45RA- and CD45RO-specific antibodies (A). Expression of CD59 was analyzed on CD45RA (B) and CD45RO (C) gated cells. One representative dot plot and histograms from 3 different experiments are shown.
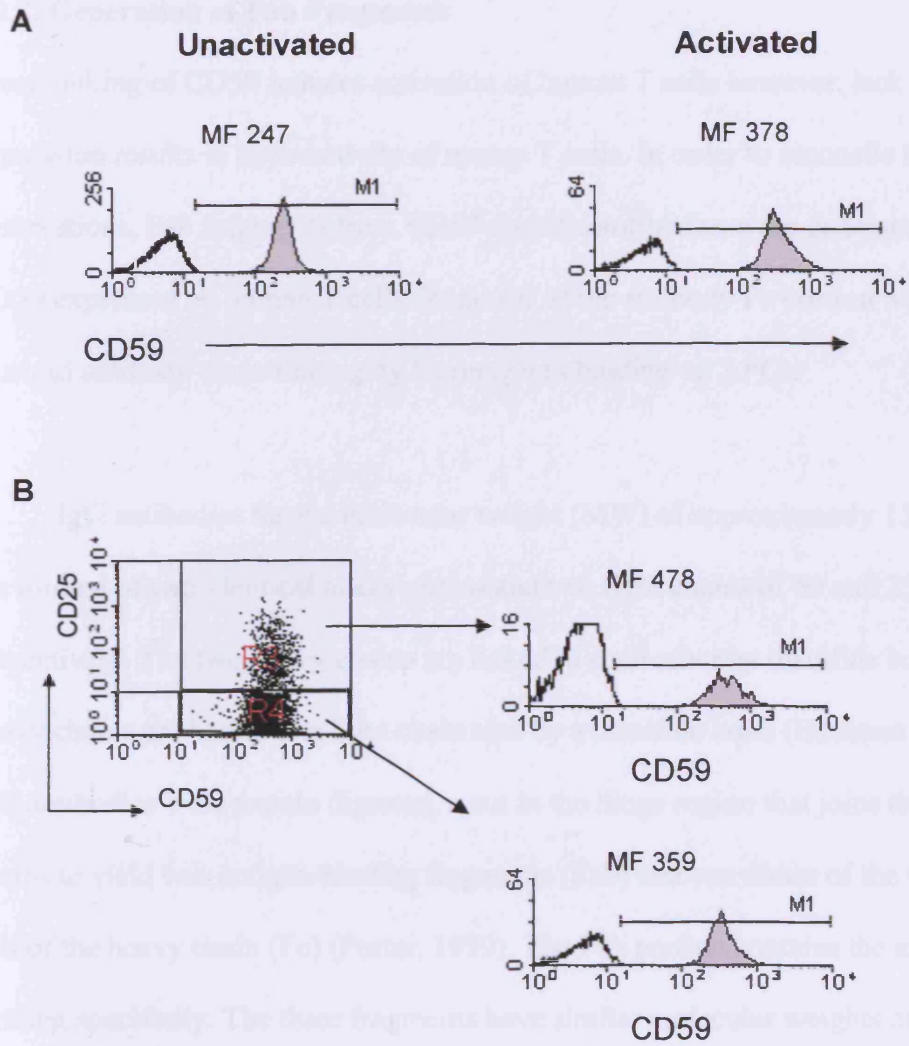
Figure 6.2. Expression of CD59 on Activated Human CD4⁺ T Cells

CD4⁺ T cells were activated with CD3-specific antibodies and APCs. 24 hours post-activation, expression of CD59 was analyzed (A). Expression of CD59 was analyzed on recently activated cells, detected by CD25 expression (B) gated cells. One representative dot plot and histograms from 2 different experiments are shown.

6.2.2. Generation of Fab Fragments

Cross-linking of CD59 induces activation of human T cells however, lack of CD59a expression results in hyperactivity of mouse T cells. In order to reconcile these observations, Fab fragments from CD59-specific antibodies were generated to block CD59 expressed on human T cells. Removal of the antibody Fc portion was necessary to avoid antibody cross-linking by Fc receptors binding on APCs.

IgG antibodies have a molecular weight (MW) of approximately 150 KDa and are formed of two identical heavy chains and two light chains of 50 and 25 KDa respectively. The two heavy chains are linked to each other by disulfide bonds and the heavy chains are bound to a light chain also by a disulfide bond (Edelman *et al.*, 1969). IgG antibodies were papain digested, a cut in the hinge region that joins the heavy chains to yield two antigen-binding fragments (Fab) and one dimer of the C-terminal half of the heavy chain (Fc) (Porter, 1959). The Fab portion contains the antigen binding specificity. The three fragments have similar molecular weights of 50 kD each.

Antibodies to human CD59 were selected based on their epitope binding regions. Antibodies MEM43 and HC1 bind to non-identical epitopes on CD59. In addition, previous work has shown that MEM43 blocks the ability of CD59 to protect against complement-mediated cell lysis while HC1 does not (Bodian *et al.*, 1997) (Figure 6.3). The antibodies, both IgG2a, were incubated with papain at a 1:25 ratio (w/w) for 18 hours. Aliquots were taken every hour and samples were analysed on a 10% SDS gel. Good antibody digestion to 50 KDa bands (Fab + Fc) was observed after ON incubation (Figure 6.4A) and was unchanged with increased concentration of enzyme (Figure 6.4B). Fab fragments were finally generated by papain incubation ON
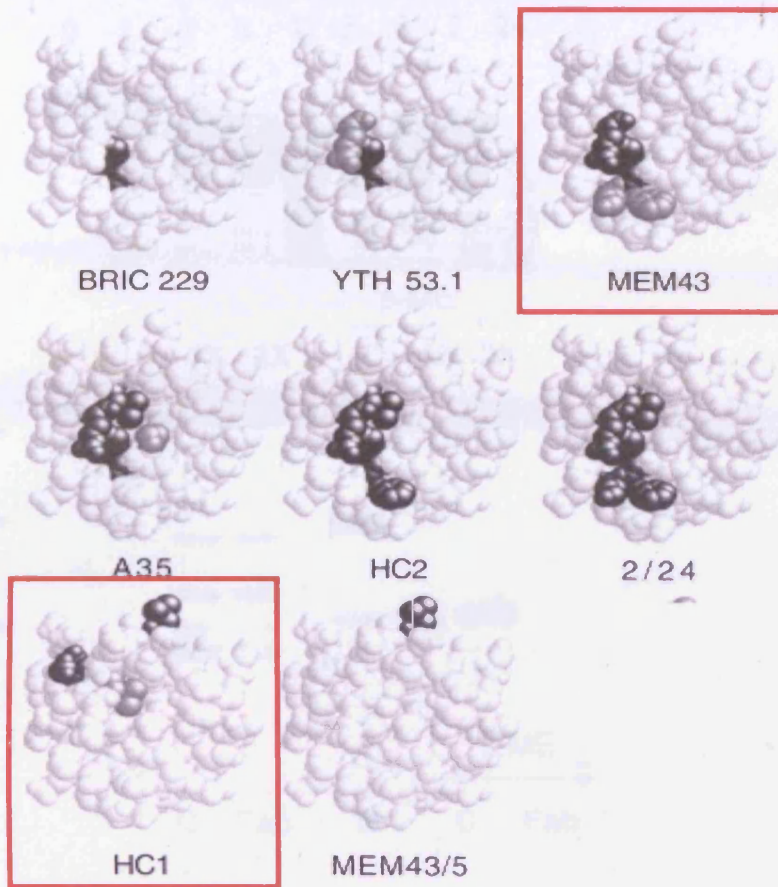
Figure 6.3. Epitope binding site of CD59-specific antibodies
Picture extracted from Bodian *et al* (1997) showing antibody binding site of different monoclonal antibodies for human CD59. Residues whose mutation disrupted the binding of each antibody are shaded black and those whose mutation led to reduce levels of binding are shaded grey. MEM43 and HC1 mAbs were selected for Fab fragment generation.
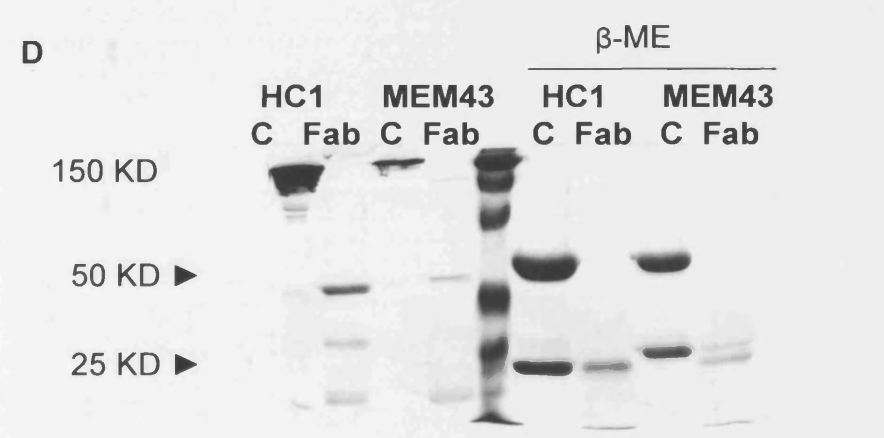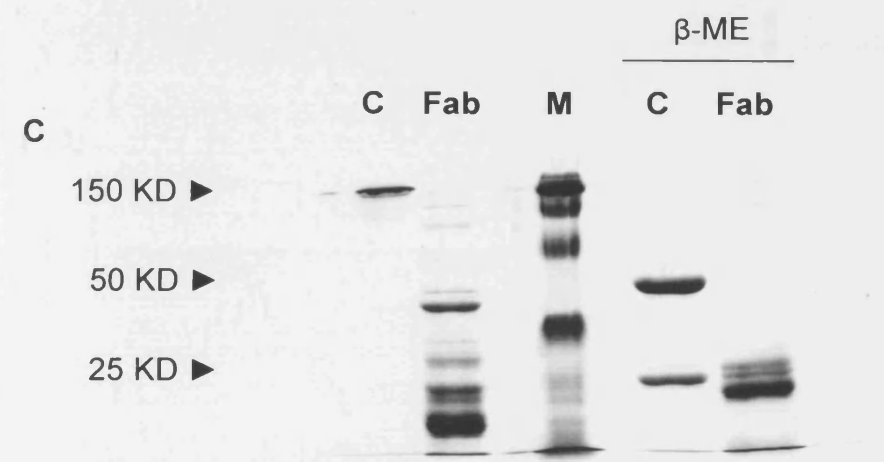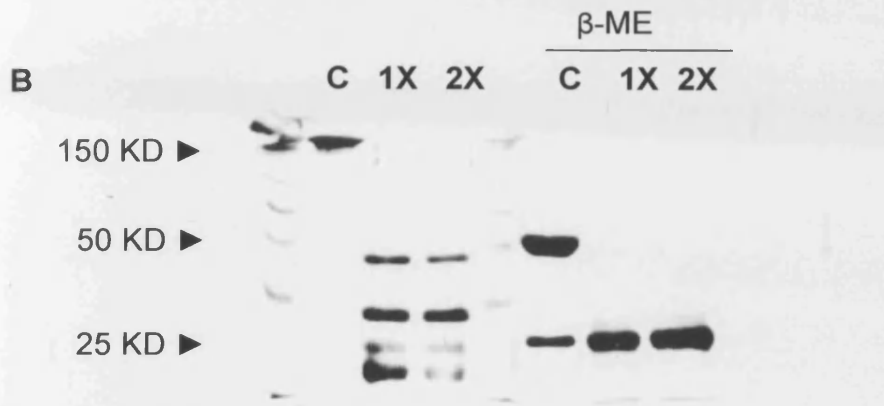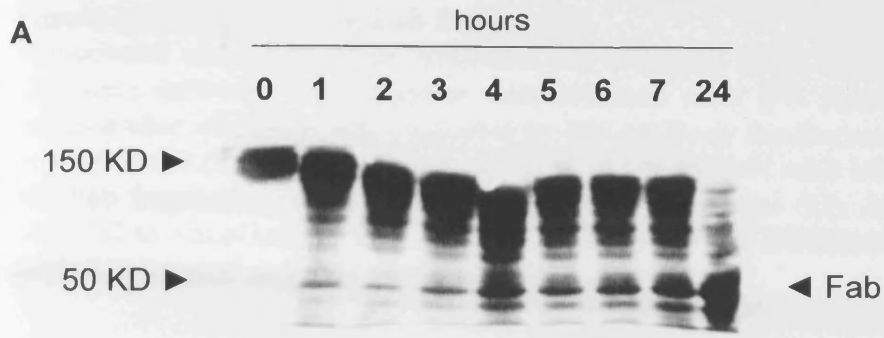
Figure 6.4. Generation of Fab Fragments

Monoclonal antibodies were incubated for different times with papain (1/25 w/w). Complete antibody fragmentation was obtained after ON incubation (A). Optimal concentration of papain was evaluated by ON antibody incubation with 1/25 w/w (1X) or 1/12.5 w/w (2X) of papain (B). Ab were incubated ON with 1/25 w/w of papain (C) and Fab fragments were purified with Hi-Trap Sepharose (D). Abs were run in 10% SDS gel to visualize the Fab band (25 KDa with and 50KDa without β-ME). As a control, untreated antibody was loaded into the gel.

at 37°C at a final concentration of 1 enzyme: 25 antibody (w/w). The Fab fragment, together with the Fc fragment, were identified as a 50 KDa band (no β-ME) or 25 KDa band (with β-ME) on a 10% SDS gel (Figure 6.4C). β-ME cleaves the disulfide bonds that link the heavy and light chains generating two bands of approximately 25 KDa. The Fc fragment was removed using a protein A column leading to a pure population of Fab fragments (Figure 6.4D).

6.2.3. Characterization of Fab Fragments

Enzymatic digestion of CD59-specific antibodies could affect their capacity to bind CD59. Fab CD59-binding was tested on human $CD4^+T$ cells by flow cytometry (Figure 6.5A). As explained above, MEM43 but not HC1 antibody blocks CD59 binding to the MAC. To functionally test the Fab fragments, complement-mediated lysis of human E was analysed in the presence of the intact antibodies or the Fab fragments. As predicted, incubation with the MEM43 antibody considerably increased complement mediated-E lysis (Figure 6.5B). Fab fragments of MEM43 were able to block E lysis, but this effect was not as pronounced as with the intact antibody. This is probably because intact antibodies are multivalent and the binding of two adjacent antigens increases the functional affinity and avidity of the antibody. Conversely, Fab fragments are monovalent and can exhibit fast off-rates and poor retention time on their targets.

As reported previously HC1 only moderately enhanced cell lysis (Figure 6.5B). The small increase observed in complement mediated lysis using HC1 may reflect the fact that CD59 is a very small molecule and bivalent HC1 antibody binding might therefore partially block the active site. Indeed, the monovalent Fab fragment of HC1
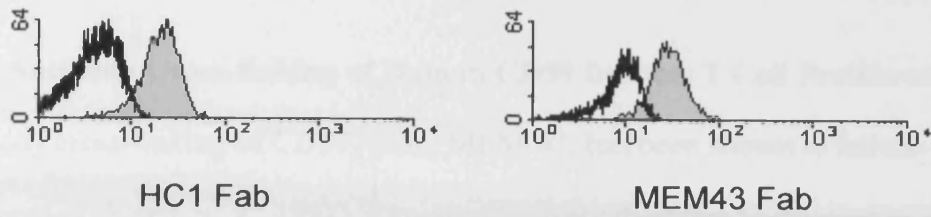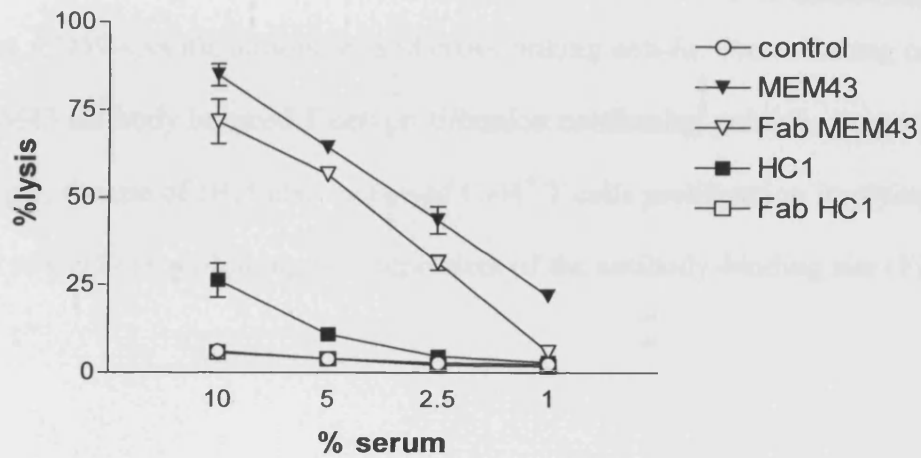
Figure 6.5. Functional Characterization of Fab Fragments
Binding of Fab fragments of CD59-specific antibodies was detected on $CD4^+$ T by flow cytometry (A). E from healthy donors were sensitized with CD55-specific antibodies and incubated with human serum (control, B). Intact and Fab fragment antibodies were added to the serum where applicable (B). E haemolysis was measured by absorbance at 415 nm. Values shown are the mean ± SD (triplicates).

did not enhance complement mediated lysis.

6.2.4. Antibody Cross-linking of Human CD59 Induces T Cell Proliferation

Antibody cross-linking of CD59, using MEM-43, has been shown to induce T cell

proliferation (Korty *et al.*, 1991). Experiments were therefore performed to confirm

this finding and to determine whether cross-linking HC1 also affects T cell

proliferation. Thus, human $CD4^+$ T cells were stimulated with CD3-specific

antibodies, CD59-specific antibodies and cross-linking anti-Ig. Cross-linking of CD59

with MEM43 antibody induced T cell proliferation confirming published reports.

Interestingly, the use of HC1 also increased $CD4^+$ T cells proliferation implying that

the effect of CD59 cross-linking is independent of the antibody-binding site (Figure

6.6).

6.2.5. Blocking of CD59 Increases $CD4^+$T Cell Proliferation

To address whether CD59 modulates T cell activation, $CD4^+$ T cells were incubated

with the Fab fragments and T cells were activated with CD3-specific antibodies and

APCs. A macrophage cell line U937 that does not express CD59, was used as an APC.

$CD4^+$ T cells were incubated with the Fab fragments, washed and then activated with

increasing concentrations of CD3-specific antibodies in the presence of APCs.

Incubation with the MEM-43 Fab fragments had no effect on T cell proliferation

whereas the HC1 Fab fragments resulted in a modest increase in proliferation at all

concentrations of CD3-specific antibodies used (Figure 6.7A). Similar results were

obtained with the use of PBMCs as APCs (Figure 6.7 B) although the effect was

significant only at low concentrations of CD3-specific antibodies. Dividing cells
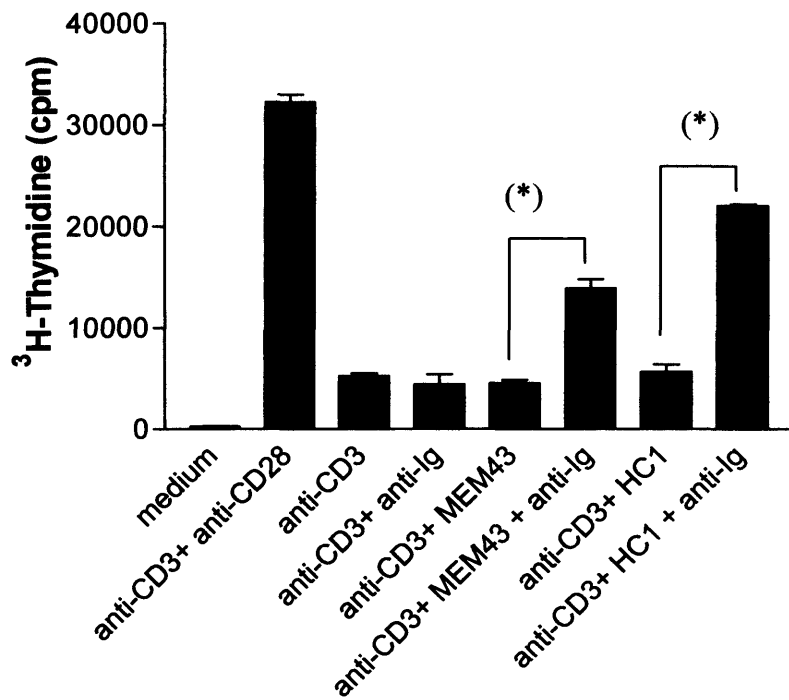
Figure 6.6. CD59 Cross-linking Induces T Cell Proliferation
Purified CD4^{+} T cells from healthy donors were incubated with MEM43 and HC1 antibodies followed by an anti-Ig antibody. T cells were activated with CD3-specific antibodies. Values shown are the mean ± SD and statistical significance (*) was evaluated using Student's T test ($p<0.05$).

express *de novo* CD59 molecules that would escape binding to the Fab fragments which were washed off prior to setting up the T cell proliferation assay. It is possible therefore that these cells continue to bind to the CD59 ligand on APCs, thereby reducing the effect of the Fab fragments. To test this possibility, $CD4^+$ T cells were stimulated in the continuous presence of antibody fragments for 3 days. Again, T cell proliferation was not affected by inclusion of the MEM-43 Fab fragments. In contrast, the proliferative response was significantly increased by the HC1 Fab fragments in a concentration-dependent manner (Figure 6.7C). As explained before, MEM-43 and HC1 antibodies bind to non-identical epitopes on CD59 suggesting that HC1 but not MEM-43 binds the active site of CD59 responsible for modulation of T cell activity. Interestingly, the HC1 binding site is not implicated in MAC regulation
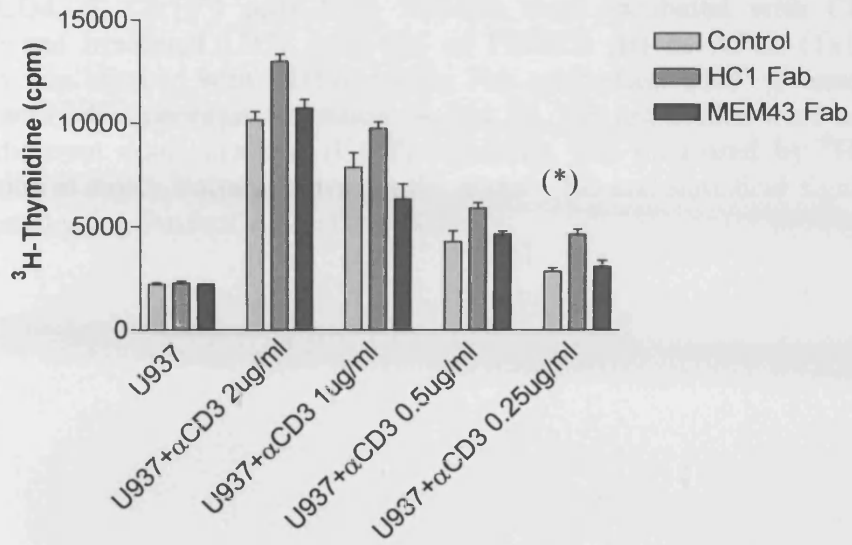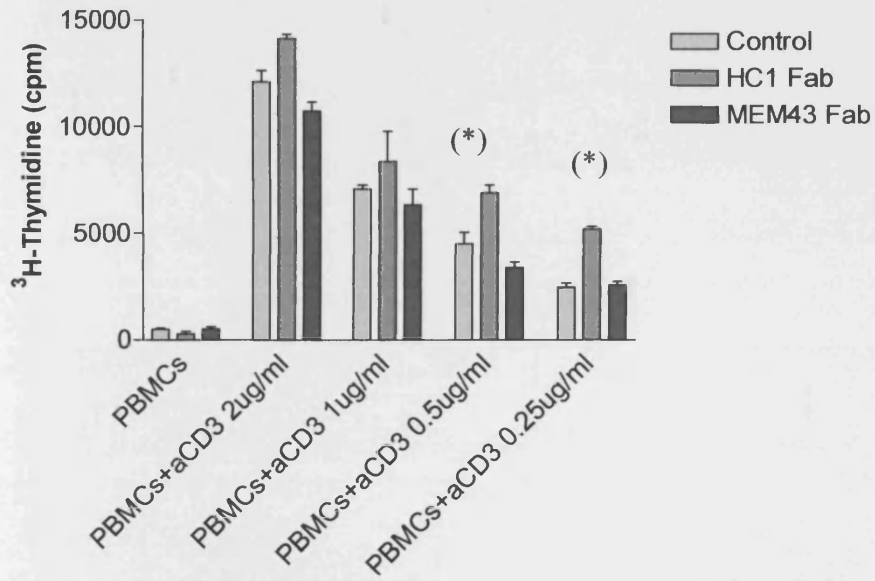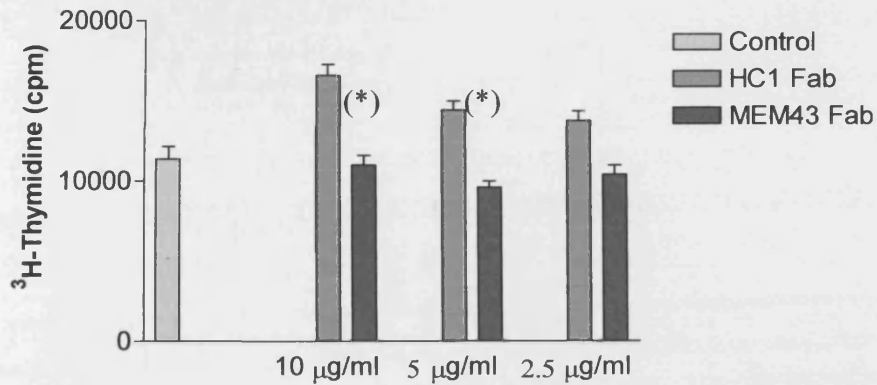
Figure 6.7. Blocking of CD59 Expression Increase CD4$^+$ T Cell Proliferation
Purified CD4$^+$ T (2x10^4) cells from PBMCs were incubated with CD3-specific antibodies and irradiated U937 cells (A) or PBMCs (B) as APCs (1x10^5). CD59 expression was blocked with CD59-specific Fab antibodies. CD4$^+$ T were incubated with Fab antibodies previous activation (A and B). Fab antibodies were added to the wells at different concentrations (C). Proliferation was measured by ^{3}H-thymidine incorporation at day 3. Values shown are the mean ± SD and statistical significance (*) was evaluated using Student's T test (p<0.05).

6.3. Discussion

CD59 is expressed on most tissues and on all circulating cells. Unlike mouse T cells where CD59a expression is barely detectable by flow cytometry (Harris *et al.*, 2003), high levels of expression were detected on human CD4$^+$ T cells. Interestingly, CD4$^+$ T cells expressing the memory marker CD45RO$^+$, expressed higher levels of CD59 than CD45RA$^+$ cells. In addition, recently activated cells expressing CD25 also up-regulated expression of CD59. This may be necessary to protect T cells from complement attack, especially activated cells present at a site of inflammation. However, based on the data described in previous chapters, up-regulation of CD59 expression may play a role in influencing T cell activation. To study this further, the capacity of selected antibodies to CD59 to modulate activation of CD4$^+$ T cells was analyzed. Two mAbs were tested, MEM-43 that targets the putative active site for complement inhibition and HC1 that binds an epitope remote from this site. Interestingly, CD59 cross-linking with both intact antibodies (MEM43 and HC1), in association with CD3 stimulation, induced T cell activation. During cross-linking experiments, activation is not induced directly by antibody binding but by protein aggregation. CD59 is a GPI-anchored protein that cannot directly trigger cell activation, but interaction with protein kinases has been proposed as a mechanism of CD59-induced T cell activation (Stefanova and Horejsi, 1991; Stefanova, 1991). Antibody cross-linking of GPI-anchored proteins generates large clusters of lipid rafts sequestering a range of surface proteins and signalling molecules that may never associate in a physiological antigen-ligand interaction. The physiological relevance of these observations is therefore questionable and raises doubts over the use of antibody cross-linking to study T cell activation.

Murine CD59a negatively modulates CD4$^+$ T cell responses *in vivo* as discussed

in previous chapters. In order to recapitulate these findings in humans, Fab antibodies

were generated to block the expression of CD59 on human T cells. Of the two

antibodies tested, only HC1 Fab fragments enhanced T cell activation. Increased cell

proliferation was moderate and only reached a 2 -fold increase with a low

concentration of CD3-specific antibodies. One plausible explanation is *de novo*

synthesis of CD59 molecules on dividing cells that are no longer blocked by

antibodies. To eliminate this possibility, CD4$^+$ T cell proliferation assays were

performed in the continuous presence of Fab antibodies. An increase in CD4$^+$ T cell

proliferation was observed in the presence of the HC1 Fab compared to control wells

and those containing the MEM-43 Fab and this difference was enhanced with

increasing concentrations of the HC1 Fab. However, the increase in cell proliferation is

minor compared to that observed in Cd59a-/- mice, and may reflect technical

limitations concerning the use of antibodies in *in vitro* assays. Interestingly, HC1

binding to CD59 does not compromise its function as a CReg suggesting that the

active site of CD59 required for T cell modulation, differs from the active site for

MAC binding. Ligand binding to different domains of CD55 elicits different biological

effects. CD55 possess 4 SCR domains and each of these modules has a specific

biological function. Brodbeck and colleagues, using recombinant DAF proteins devoid

of individual SCRs, demonstrated that the regulation of the classical pathway of

complement resides in the SCR2, SCR3 domains while, blocking of the alternative

pathway, also requires the SCR4 domain (Brodbeck *et al.*, 1996). The SCR1 domain

has recently been described to bind the CD97 molecule, a non-complement ligand for

CD55.

The Effect of Antibody Binding. Activation vs Blocking?

As discussed above, Fab binding to CD59 enhances T cell activation depending on the antibody clone used. Fragmented HC1 antibody-binding to the non-complement related active site of CD59, enhances $CD4^+$ T cells proliferation while Fab fragment binding to the complement related active site of CD59 (MEM-43) has no effect. Antibody Fab fragments have been successfully used by several investigators to modulate T cell responses (Brown *et al.*, 2003; Haspot *et al.*, 2002; Krummel *et al.*, 1996; Perrin *et al.*, 1999; Vanhove *et al.*, 2003). Fab antibodies to CD28 have been shown to reduce T cell proliferation by blocking its costimulatory function (Haspot *et al.*, 2002; Krummel *et al.*, 1996; Perrin *et al.*, 1999; Vanhove *et al.*, 2003). However, the effect of Fab binding may differ with distinct antibody clones. While Fab fragments with high affinity blocked CD28 function, Fab fragments with low binding affinity showed little or no effect on T cell proliferation (Vanhove *et al.*, 2003). In the studies performed here, a low binding affinity to CD59 could explain the moderate effect on $CD4^+$ T cell proliferation observed with HC1 Fab fragments. Thus, it becomes impossible to discriminate between blocking and activating effects of HC1 and MEM43 binding. There is a general consensus that Fab antibodies block protein function however, it appears that inhibition depends on the antibody binding site rather than the antibody valence. Monovalent binding (Fab) as well as intact antibodies to CD3 has been shown to induce T cell activation and IL-2 production in human T cell clones (Ledbetter *et al.*, 1986; Peacock *et al.*, 1991; Tamura and Nariuchi, 1992). This suggests that, when the function of the molecule is unknown, it becomes difficult to predict the effect of the antibody binding. Thus, while the data presented in this chapter imply that blocking CD59 does indeed promote T cell activation, solid conclusions cannot be drawn using the methods (incubation with intact antibodies and

Fab fragments) utilised so far. Better methods for blocking CD59 expression on human T cells are now needed. With this in mind, silencing of CD59 using siRNA offers an alternative to antibody blocking. The use of siRNA has proven to be a valid approach for sequence-specific suppression of gene expression and therefore for loss of function studies. Such experiments should allow solid conclusions to be drawn over whether CD59 negatively or positively modulates human T cell responses.

Chapter 7- Final Discussion

7.1. CD59a Modulates Immune Responses

Complement plays a key role in linking innate and adaptive immunity. While complement activation during the early events of the immune response provides a rapid and efficient means for opsonising or killing pathogens and triggers inflammation, it also plays a role in adaptive immunity, particularly in the humoral immune response. Seminal studies by Pepys and colleagues showed that C3 played a role in antibody production (Feldmann and Pepys, 1974; reviewed in Pepys, 1972). This link between complement and adaptive immunity was further investigated and defined by several groups some twenty years later, who demonstrated that antigen opsonised with C3 fragments potentiated the immune response by crosslinking complement receptor 2 and surface IgM in a signalling complex on the B cell (Matsumoto *et al.*, 1991; reviewed in Carroll, 2004). Complement activation is a potentially dangerous system and is therefore very strictly regulated by CReg. MAC has been implicated in the pathogenesis of several autoimmune and inflammatory diseases (reviewed in Nangaku, 2003). Over-activation of complement in the absence of CD59a, has been shown to exacerbate tubulointerstitial damage after renal Ischemia-reperfusion injury (IRI) (Turnberg *et al.*, 2004). In this model, Cd59a-/- mice exhibited increased tubular injury, neutrophil recruitment and MAC deposition in tubular epithelial cells. Inhibition of the terminal pathway of complement protected mice from renal IRI injury suggesting that MAC is critical in complement mediated renal post-ischemic injury (Zhou *et al.*, 2000). A similar role for CD59a and MAC was suggested in a mouse model of accelerated nephrotoxic nephritis (ANTN) (Turnberg *et*

al., 2003). In a model of antigen-induced arthritis (AIA), Cd59a-/- mice exhibited high

MAC deposition and joint pathology with increased cartage destruction, bone erosion

and cellular infiltration compared to control mice (Williams *et al.*, 2004). In addition,

expression of human CD59 in inflamed joints in rheumatoid arthritis was reduced

compared with non-inflamed tissues suggesting that reduced regulation of the MAC is

relevant for the development of arthritis (Konttinen *et al.*, 1996). Thus, CD59a can

modulate immune responses by controlling complement activation and MAC

formation. CD59a, by blocking the formation of the MAC, will impinge on the

magnitude of the immune response generated. MAC deposition has been shown to

amplify inflammatory responses by direct and indirect mechanisms. A number of

studies have shown that the deposition of a sub-lytic concentration of MAC on

endothelial cells triggers cell activation and the release of inflammatory mediators such

as MCP-1, IL-8, and RANTES (Kilgore *et al.*, 1995; Ward, 1996). Simultaneously,

MAC can create complete transmembrane channels leading to osmotic lysis of the cell.

Necrotic cells and the intracellular material released from them can bind complement

components (e.g. C1q, C3 and C4) and amplify complement activation and the

inflammatory response (Jiang *et al.*, 1992; Gaipl *et al.*, 2001) (Figure 7.1A, B and C).

The work presented in this thesis indicates that CD59a, which serves to downregulate

activation of the complement cascade, also has the effect of downregulating T cell

responses (Figure 7.1D)

The studies described in this thesis, demonstrate a novel role for the membrane

CReg CD59a in modulation of T cell responses. Virus- specific $CD4^+$ T cell responses

were increased in Cd59a-/- mice compared to control mice. In addition, higher
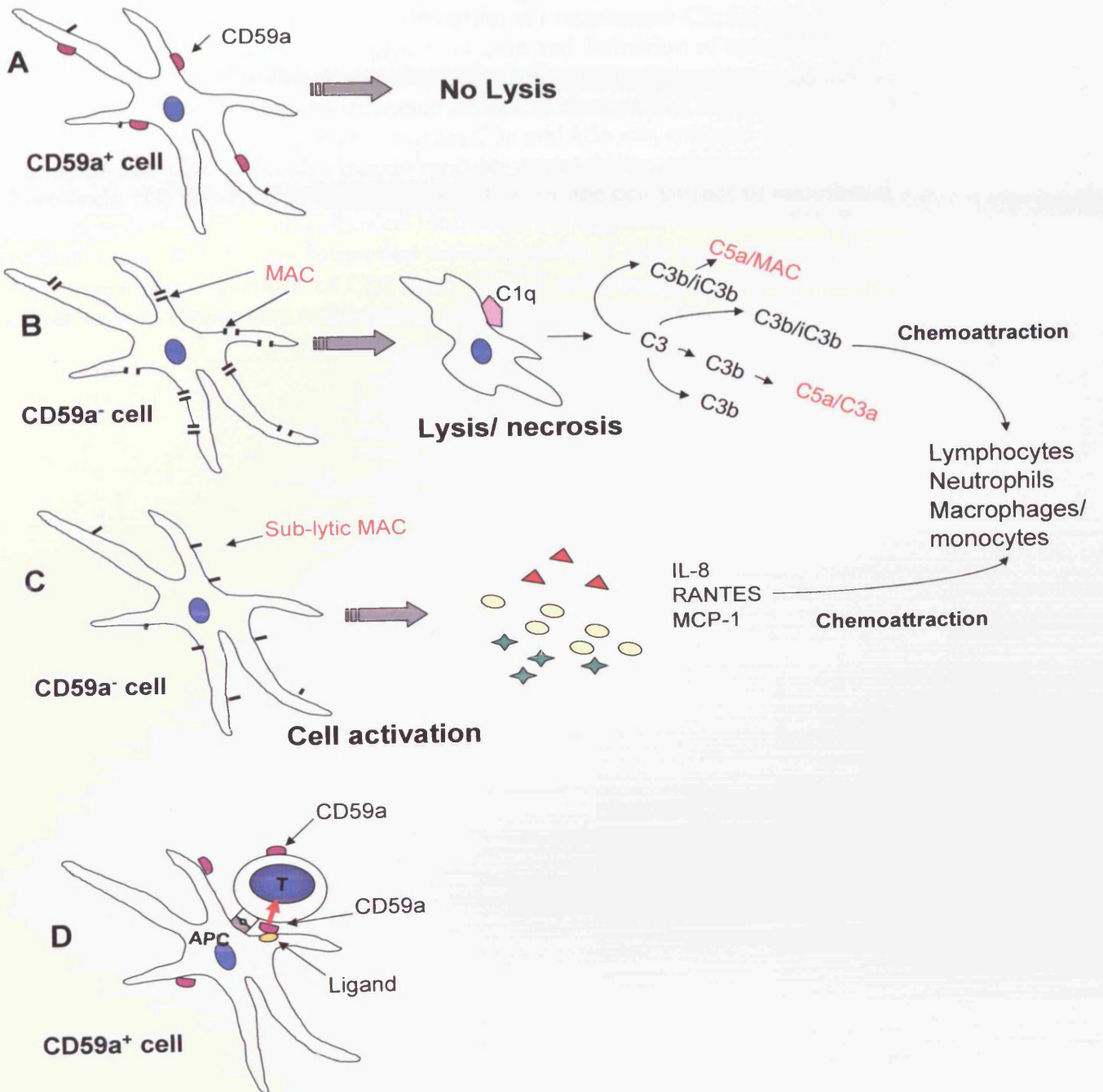
Figure 7.1. Interplay Between the Innate and Adaptive Immune System in the Absence of CD59a

CD59a protects cells from MAC formation and cell lysis (A). Lack of CD59a increases MAC deposition on cell membrane. (B) MAC opens a pore in the membrane of targeted cells resulting in cell lysis. Deposition of complement C3, C4 and C1q on necrotic cells exacerbates complement activation and formation of complement component that can play important roles in the inflammatory response. Ligation of CD11b with C3b/iC3b leads to increased adhesion, chemotaxis, and phagocytosis of both neutrophils and macrophages whereas C3a and C5a can enhance expression of neutrophil-adhesion molecules induce vascular permeability and attract leukocytes by chemotaxis. (C) Sub-lytic concentration of MAC on the cell surface of endothelial cells increases expression of adhesion molecules and induces secretion of IL-8, RANTES and MCP-1 chemoattractant for neutrophils, T cells and monocytes respectively. (D) Expression of CD59a on $CD4^+$ T cells negatively modulates T cell activation in a complement independent manner.

frequencies of lymphocytes were present at the main site of primary infection in Cd59a -/- mice. The effect of CD59a deficiency on CD4$^+$ T cell proliferation was complement independent since inhibition of complement using sCR1 did not reverse the effect of CD59a-deficiency on CD4$^+$ T cell proliferation. Furthermore, enhanced virus-specific CD4$^+$ T cell proliferation was also observed in Cd59a-/- mice backcrossed onto a C3-deficient background. These experiments rule out the possibility that a complement component ligates CD59a on T cells to mediate this effect.

It is likely that the complement dependent and independent effects of CD59a expression act in conjunction to negatively modulate the immune response. In the influenza virus model described in Chapter 4, increased lung inflammation in Cd59a-/- mice correlated with a complement-dependent infiltration of neutrophils and a complement-independent infiltration of IFNγ-producing CD4$^+$ T cells. Activated neutrophils that have been recruited at the site of inflammation by complement component derived and MAC-mediated inflammatory mediators release cytokines and chemokines that induce recruitment of T cells as well as other immune cells (Bonder *et al.*, 2004; reviewed in Scapini *et al.*, 2000). Thus, absence of CD59a may boost the CD4$^+$ T cell response in two ways; Firstly, a more robust innate immune response induced in the absence of CD59a could serve to amplify the subsequent T cell response and, secondly removal of the inhibitory effect of CD59a expression on the T cells themselves would also promote their expansion. In Cd59a-/- mice, enhanced neutrophil recruitment will therefore result in more T cells and, simultaneously, these T cells will proliferate more by CD59a complement-independent mechanism. It is notable that, in the models of renal IRI, ANTN and IAI discussed above, high cellular infiltration was observed in Cd59a-/- mice (Turnberg *et al.*, 2004; Turnberg *et al.*,

2003; Williams *et al.*, 2004). This could be due, in part, to the lack of T cell modulation by CD59a. In neither of these studies was cellular infiltration assessed in the absence of complement activation. In order to discriminate the indirect effect of CD59a deficiency on T cell responses which might result from amplification of complement activation and direct effects of CD59a on T cell activation, transgenic mice where CD59a is expressed only on T cells would be useful. This technique has been successfully performed to generate several transgenic mice (Carrasco *et al.*, 1997; Galkina *et al.*, 2003) and could be used to provide a better understanding of the role of CD59a in modulating CD4$^+$ T cell responses.

7.2. Possible Mechanisms for Modulation of T Cell Responses by CD59a

In this thesis, Cd59a-/- mice have been shown to exhibit enhanced CD4$^+$ T cell responses to virus. T cell hyper-response was not abrogated by complement inhibition suggesting that CD59a negatively modulates CD4$^+$ T cell activity in a complement-independent manner. As discussed previously, the role of CD59a as a CReg is well described but little is known about its direct role on T cell activation. As shown in Chapter 3, proliferation assays performed *in vitro* demonstrated that CD4$^+$ T cells, lacking CD59a expression, proliferated more vigorously in response to stimulation with CD3 specific antibodies and APCs. Interestingly, no difference was observed in proliferation when the T cells were stimulated with CD3- and CD28-specific antibodies indicating that APCs were required to observe the enhanced proliferation in Cd59a -/- T cells. These data implied that CD59a on T cells normally engages with a ligand on APCs, a hypothesis that was supported by the observation that a CD59a-

IgG$_{2a}$ fusion protein bound to macrophages, DCs and B cells. Experiments performed

in this thesis could not find any negative effect of CD59a engagement on APC

function; thus downmodulation of T cell activity by CD59a engagement appears to be

through inducing a negative signal in the T cell rather by transmitting a negative signal

that results in downmodulation of the APC. The question of how a GPI-anchored

protein modulates T cell activation therefore arises.

The mechanism with which CD59, as well as other GPI-anchored proteins,

delivers intracellular signals is not yet clear. Since the GPI anchor only transverses the

outer leaflet of the membrane bilayer, it is likely that signalling will depend on

association with other molecules. GPI-anchored proteins co-precipitate with p56lck

and p59Fyn kinases (Shenoy-Scaria, 1992; Stefanova et al., 1991). Thus it is possible

that association of GPI protein with the Src family of protein kinases leads to cell

signalling. The effect of GPI-anchored proteins on T cell activation may be a reflection

of their presence in lipid rafts and their accumulation in the immunological synapse.

Engagement of the TCR with MHC-peptide complex results in macromolecular

rearrangements of the T cell membrane resulting in the formation of an organized

structure called the "immunological synapse" (IS). This structure is formed within 5-

30 minutes of TCR engagement and is associated with reorganization of the actin

cytoskeleton and segregation of adhesion and TCR-associated molecules into two

major components, a central and peripheral supramolecular activation cluster (cSMAC

and pSMAC). The TCR-CD3 complex, together with CD28, PKCΘ, Lck and Fyn, are

found in the cSMAC while LFA-1, talin and CD4 are present in the pSMAC. Upon

TCR stimulation, many signalling proteins selectively concentrate in membrane

microdomains called lipid rafts e.g. ZAP-70, phospholipase Cγ1 (PLCγ1), Vav

Rac/CDC42 exchange factor and the protein kinase Cq (PKCq), and these rafts are thought to accumulate mainly within the cSMAC (Burack *et al.*, 2002; Montix *et al.*, 1998; reviewed in Alonso and Millan, 2001). If rafts do indeed act as platforms for T cell signalling, then it is possible that this represents a way through which GPI-anchored proteins could positively or negatively influence T cell activation. It is believed that maintaining signalling molecules in distinct heterogeneous microdomains within the plasma membrane is a mechanism through which T cells orchestrate a diverse and controlled response (Gomez-Mouton *et al.*, 2001; reviewed in Germain and Stefanova, 1999). In other words, a protein A is separated from protein B and protein C in lipid rafts. Upon T cell activation, changes in the organization of the plasma membrane and its constituents, facilitate the rearrangement and redistribution of raft-associated proteins, thereby allowing contact of protein A with protein B but remaining separated from protein C. Heterogeneity in lipid rafts composition may be a mechanism by which discrete signalling pathways can be differentially activated during an immune response by changes in the lateral mobility of enzymes, substrates, and adaptor proteins within the plasma membrane. Marmor and colleagues showed that activation of T cells with immobilized specific antibodies to TCR and GPI-anchored proteins (CD48, Thy-1, Ly6-A, TSA-1 and Qa-2) suppressed cell proliferation but not IL-2 release or cytotoxicity. Despite the production of IL-2, GPI-immobilized cells failed to signal through the IL-2R resulting in a profound reduction of IL-2-induced cell growth. Three chains participate in the formation of the IL-2R (IL-2Rα, β and γ) and are mainly partitioned into lipid rafts. Investigators found that IL-2R on these cells lacked the expression of the IL-2Rβ that is known to be associated to the protein tyrosine kinase JAK1. GPI-anchored proteins may participate in the lateral mobility of IL-2R chains necessary for hetero-trimerization of the IL-2R. Immobilization of GPI-

anchored proteins with antibodies could prevent the trafficking of some of the chains and, therefore, the assembly of the subunits. This last hypothesis of raft heterogeneity and lateral mobility of GPI-anchored proteins may explain why both cross-linking CD59 or CD59a and deleting CD59a expression on T cells promotes T cell proliferation. When cross-linked mouse CD59a or human CD59 proteins are not freely and selectively migrating within the plasma membrane but are forced to aggregate and associate with other proteins eliciting dramatically different immunological effector functions compared to natural ligand binding.

Based on the discussion above, expression of CD59 in membrane microdomains raises the possibility that CD59 may interfere with the formation of the immunological synapse. No studies exploring this role have been described. Expression of CD59a on T cells is very low and cannot be easily detected by immuno-fluorescence techniques. In an attempt to study CD59a distribution during formation of the immunological synapse, murine T cell lines were transfected to over-express CD59a. As a result, expression of CD59 was not restricted to lipid rafts but also was expressed in non-rafts fractions making interpretation difficult. On human T cells, cross-linking of CD59 showed patchy distribution of CD59 by confocal microscopy, co-localization with actin filaments and phosphotyrosine enrichment in the lipid raft patches (Deckert *et al.*, 1996; Harder and Simons, 1999). At the moment, no studies of membrane CD59 distribution under physiological condition of T cell stimulation have been reported.

Clearly, many questions regarding the role of CD59 on T cells remain to be answered. As discussed in Chapter 5, CD59a appears to have a direct negative effect on T cell activation. However, due to technical limitations, it was impossible to

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confirm this theory. To induce stronger signals for measurement of very early events in T cell activation (Ca^{2+} flux and protein phosphorylation) it will be useful to cross Cd59a-/- mice with CD4$^+$ TCR transgenic in order to allow the experiments to be carried out using high frequencies of antigen-specific T cells. This methodology will give A) a stronger signal following T cell activation and B) simultaneous T cell activation through TCR and CD59a ligand. Identification of the natural ligand for CD59a will also prove extremely useful for studying modulation of T cell activity by CD59a. At the moment this is an area of intense research in the laboratory.

7.3. Human CD59 as a Therapeutic Target

7.3.1. Manipulation of immune responses

Appropriate stimulation of the immune system is crucial for successful resolution of infection/ damage. Turning the immune response off after an infection is controlled is equally important since hyper-stimulation of the immune system can lead to uncontrolled inflammation. Due to the complex and multigenic processes underlying T cell activation, several pathways can be targeted to reduce T cell responses such as antigen recognition, co-stimulation and regulation (Daoussis *et al.*, 2004; Krinzman *et al.*, 1996; Latchman *et al.*, 2001). Glucocorticoids and anti-histamines are currently used for controlling immune responses but they are largely palliative and non-specific potentially causing immunosuppression. CD59 prevents complement-mediated cell lysis but does not block opsonisation or anaphylatoxin production. In addition, CD59 negatively modulates T cell responses but still allows the mounting of an adequate immune response. Thus the use of CD59 as a therapeutic target may be advantageous since the effects on the immune responses would be expected to be subtle, allowing modulation of the immune response without completely switching off host defenses.

Administration of membrane-targeted recombinant rat CD59 (sCD59-APT542) in mouse and rat, was successfully used as a therapeutic agent for AIA. Animals treated with sCD59-APT542 showed a significant decrease in joint inflammation with minimal synovial hyperplasia, cartilagenous/bony erosions and less mononuclear cell infiltration (Fraser et al., 2003; Williams et al., 2004). It is important to note however, that this recombinant protein is missing the GPI-anchored portion that may be relevant for direct modulation of the T cell response.

Recently, a novel recombinant human CD59 soluble protein (rhCD59-P) has been described to protect the erythrocytes of patients with PNH from complement-mediated cell lysis (Hill et al., 2006). In this study, CD59 was modified with a novel synthetic membrane-anchored peptide that efficiently attaches to cell membranes for at least 3 days in vitro. This new recombinant CD59 protein might also have potential for the treatment of uncontrolled inflammatory responses.

7.3.2. Recombinant CD59 as a strategy for immune-modulation

Inhibition of immune responses during virus infection may result in poor control of virus replication and persistence of disease. At the same time, over-reaction of the host's immune system may be accompanied with pathology as described for influenza virus infection. In this case, the use of recombinant CD59 (rCD59) may be beneficial to the host by reducing lung inflammation but still allowing the generation of an adequate immune response. Infection of Cd59a-/- mice with influenza virus resulted in high lung inflammation with increased numbers of infiltrating neutrophils and CD4$^+$ T cells compared to WT mice. It will be of interest to test whether administration of rCD59 can limit the immunopathological consequence of complement activation and T

cell hyperactivity in these mice after influenza infection. For this purpose, rCD59 could be administrated intranasally to target the respiratory tract.

7.3.3. Recombinant CD59 for Tumor Immunotherapeutic

The strategy employed to treat a particular disease will depend on whether the mounting of an immune response is beneficial or detrimental to the patient. In the case of tumour immunotherapy, it is clearly beneficial to promote tumour-specific immune responses. Adoptive transfer of T cells has been intensively investigated for treatment of cancer (Rosenberg *et al.*, 1988a; Rosenberg *et al.*, 1988b; reviewed in Dudley and Rosenberg, 2003). Adoptive T-cell therapy using antigen-specific CD8^{+} T cells for cancer treatment has been attempted with some degree of success (reviewed in Dudley and Rosenberg, 2003). The trials performed so far indicate that a major obstacle to the success of effective lymphocyte transfer therapies for cancer is failure of the transferred cells to persist in the recipient (reviewed in Dudley and Rosenberg, 2003). It may be possible to enhance anti-tumor T cell survival / persistence by silencing molecules known to have a negative effect on T cell proliferation / survival. CD59 may represent one such molecule. RNA interference using short, double-stranded RNA (small interfering RNA, or siRNA) is currently used for suppressing gene expression in mammalian cells and, since siRNA has already been shown to effectively silence expression of different genes on PBMCs (Coburn and Cullen, 2002; Qureshi *et al.*, 2006), it may prove possible to use this technology for the silencing of CD59 expression on T cells (Elbashir *et al.*, 2001; Ge *et al.*, 2003; reviewed in Dorsett and Tuschl, 2004; Dykxhoorn *et al.*, 2003). Lymphocytes have several features which make them attractive for gene therapy. The cells are readily available from peripheral blood as a single cell suspension and are easily manipulated in tissue culture, expanded

by IL-2 allowing gene insertion, selection and testing prior to their return to the patient. In summary, the strategy of silencing CD59 expression on T cells to enhance immunity has of great potential for the treatment of different diseases.

7.4. Conclusion

In this thesis, I demonstrated a new role for CD59a distinct from its common role as a CReg. The novel finding that CD59a has a role in CD4$^+$ T cell activation is of utmost importance to the field of immunology. I showed that murine CD59a modulates antivirus CD4$^+$ T cells responses, an effect that was independent of complement activation. The exact mechanism by which CD59a controls CD4$^+$ T cells activity remains unclear but it is believed that, upon binding to an unknown ligand on APCs, CD59a directly modulates T cell activation. This study also demonstrated that CD59a can modulate immune responses in a complement-dependent manner. Influenza-infected Cd59a-/- mice exhibited enhanced numbers of lung-infiltrating neutrophils compared to WT mice. This effect was complement-dependent suggesting that CD59a, by blocking MAC deposition, also modulates the innate immune response to virus. Several questions however, remain to be answered. Identification of the ligand is imperative for full understanding of the mechanism by which CD59a modulates CD4$^+$ T cell responses. In addition, in order to recapitulate the mouse findings in the human system, more appropriate methodology will have to be implemented as discussed in Chapter 6. The results of these studies will enable the possibility of manipulating immune activity through targeting CD59 to be more fully explored.

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Cutting Edge: Murine CD59a Modulates Antiviral CD4⁺ T Cell Activity in a Complement-Independent Manner[1]

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CD59 blocks formation of the membrane attack complex of complement by inhibiting binding of C9 to the C5b-8 complex. To investigate a role for CD59 in promoting T cell responses, we compared T cell activation in CD59a-deficient (Cd59a⁻/⁻) and wild-type (WT) mice after in vitro stimulation and after infection with rVV. Virus-specific CD4⁺ T cell responses were significantly enhanced in Cd59a⁻/⁻ mice compared with WT mice. Similarly, Cd59a⁻/⁻ T cells responded more vigorously to in vitro stimulation with CD3-specific Abs compared with WT mice. This effect of CD59a on T cell proliferation was found to be complement-independent. Collectively, these results demonstrate that CD59a down-modulates CD4⁺ T cell activity in vitro and in vivo, thereby revealing another link between complement regulators and T cell activation. The Journal of Immunology, 2005, 175: 7098–7102.

H uman CD59 is a 18–20 kDa GPI-anchored protein expressed in all circulating cells and in most tissues (1). In common with other GPI-anchored proteins, it is found in membrane microdomains, lipid rafts, which serve as platforms for Ag receptor signaling complexes in lymphocytes. CD59 acts as a complement (C)[3] regulator by inhibiting the formation of the membrane attack complex (2). Others have suggested that CD59 also acts as a costimulatory molecule for T cell activation. Ab-mediated cross-linking of CD59 on PMA-treated human T cells caused enhanced proliferation and IL-2 production (3).

To explore the role of CD59 in T cell activation in vivo, we examined T cell function in mice lacking the species analog, CD59a (4). T cell activity was compared in Cd59a⁻/⁻ and Cd59a⁺/⁺ (wild-type; WT) mice after in vitro stimulation and after infection of mice with recombinant vaccinia virus (rVV). CD8⁺ T cell responses were unaltered in the absence of CD59a; however, CD4⁺ T cells displayed enhanced proliferation to several stimuli both in vivo and in vitro. C inhibition did

not influence the enhanced responses, indicating that CD59a modulates CD4⁺ T cell activation independent of C.

Materials and Methods

Mice

C57BL/6 (H-2ᵇ) mice (WT) were obtained from Harlan. B6.129-Cd59aᵗᵐ¹ᴮᵖᵐ (Cd59a⁻/⁻) mice were generated as described previously (4) and backcrossed onto the C57BL/6 background for eight generations. Cd59a⁻/⁻ mice were intercrossed with the B6.129S4-C3ᵗᵐ¹ᶜʳ (C3⁻/⁻) mice (5) also on the C57BL/6 background. Experiments were performed in compliance with Home Office regulations.

Cell culture

Yac-1 cells, T cells, and V8E, a mouse CD4⁺ T cell hybridoma (6) (provided by Dr. Annette Oxenius, University of Zurich, Zurich, Switzerland), were maintained in RPMI 1640 medium supplemented with 10% FCS, penicillin-streptomycin, L-glutamine, and 2-ME. V8E cells were transfected with CD59a using standard methods.

Infection with rVV and determination of antivirus response

rVV expressing the gp of lymphocytic choriomeningitis virus (LCMV) has been described previously (7). Mice received injections i.p. with 50 μl of rVVGP at 10⁸ PFU/ml. At day 3 and 8 after infection, ovaries were harvested for virus titers, and immunostaining and spleens were harvested for CTL assays and T cell proliferation assays. For memory responses, spleens were harvested 6 wk after infection, and CTL assays and CD4⁺ T cell proliferation assays were performed.

Fluorescence staining

Cy5-conjugated Abs were used for CD4 staining (Caltag Laboratories). Cells were incubated with 5 μg/ml mAb for 30 min, washed, and resuspended in FACS buffer. IFN-γ staining was performed using IFN-γ-FITC mAbs (BD Pharmingen) after incubating ovary-derived lymphocytes for 4 h at 37°C in the presence of ionomycin (1 μg/ml), PMA (20 ng/ml), and monensin (3 μM) (Sigma-Aldrich). CFSE staining (Molecular Probes) was conducted by incubating the cells for 10 min at 37°C with 0.5 μM CFSE. In all cases, cells were resuspended in FACS buffer and analyzed by FACS (FACSCalibur; BD Biosciences).

Immunoprecipitation and Western blotting

Immunoprecipitation was performed as described previously (8). Lysates of mouse CD4⁺ or CD8⁺ T cells were incubated with 10 μg of mouse mAb against CD59a (mCD59.4) (9) and 50 μl of protein A-Sepharose beads for 2 h at 4°C. The immunoprecipitates were washed in lysis buffer and boiled in nonreducing SDS-PAGE sample buffer. Beads were removed by centrifugation,

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and supernatants were resolved on nonreducing 12% SDS-PAGE and transferred to nitrocellulose. The membrane was probed with the CD59a-specific mAb mCD59.1 as described previously (9).

T cell proliferation

CD4$^+$ and CD8$^+$ T cells from single-cell suspensions of splenocytes were purified by positive MACS MicroBeads selection (Miltenyi Biotec). To produce APCs, spleen cells were panT depleted (Dynabeads; Dynal Biotech) and irradiated with 2400 cGy CD4$^+$, or CD8$^+$ cells (2×10^4 cells) were incubated with 10^5 APCs and 1 μg/ml anti-CD3 mAb in a 96-multiwell (MW) plate. Cell proliferation was assessed by thymidine incorporation or CFSE FACS analysis at day 3. For anti-CD28 and anti-CD3 mAb stimulation, 10^5 CD4$^+$ T cells were incubated in a 96-MW plate with 2 μg/ml anti-CD28 and 2 μg/ml anti-CD3 (Leinco Technologies). Vaccinia-specific CD4$^+$ T cell proliferation was performed by incubation of 10^5 CD4$^+$ T cells with 6×10^5-irradiated splenocytes and 2.5 μg/ml P13 peptide (GLNGPDIYKGVYQFKSVEFD) (LCMV-GP, I-A^b) or P61 peptide (SGEGWPYIACRTSVVGRAWE) (LCMV-NP, I-A^b). CD8$^+$ T cell proliferation was conducted against the D^b-restricted peptide gp33. Cells were incubated for 6 days, and thymidine was added for the last 18 h.

Exogenous incorporation of CD59a

CD59a was purified as described previously (10). CD4$^+$ T cells (5×10^6 cells) were incubated with 5 μg of CD59a for 20 min at 37°C to permit incorporation via the GPI anchor. Cells were then washed and incubated for another 2 h to allow migration of CD59a into lipid rafts (11).

CTL assay

Spleen cells (4×10^6 cells) were stimulated in vitro with 1×10^6 gp33 (KAVYNFATM) (LCMV-GP, D^b) peptide-loaded (10^{-5} M) splenocytes, and IL-2 was added at day 2. After 1 wk, cells were restimulated with 1×10^6 gp33 splenocytes and IL-2 (10 U/ml). rVV-specific CTL activity was measured 5 days later as described previously (12).

Virus titers

rVVGP titers were determined in ovaries at day 3 and 8 postinfection. Ovaries were homogenized and incubated on a monolayer of TK$^-$ cells as described previously (13).

CD59a cross-linking and IL-2 ELISA

V8E, a mouse CD4$^+$ T cell hybridoma negative for CD59a expression by FACS, was transfected with CD59a as described (9). Purified CD4$^+$ T cells from Cd59a$^{-/-}$ or WT mice or V8E cells, untransfected or transfected with CD59a, were incubated with mCD59.1 or isotype-control mAb for 15 min at 4°C. After washing, cells were plated at 10^5 cells/well in triplicate in a 96-MW plate. Where appropriate, 5 μg/ml F(ab')$_2$ rabbit anti-rat IgG Ab (Serotec) was added to the wells to cross-link (3). Cells were activated with 0.5 ng/ml PMA and incubated at 37°C. After 18 h, IL-2 was measured by ELISA according to the manufacturer's instructions (BD Pharmingen).

C inhibition

For inhibition of C activity in vitro, 1 μg/ml recombinant human soluble CR1 (sCR1; gift from T Cell Sciences) was added to each well of a 96-MW plate. Cell proliferation was assayed after 3 days by thymidine incorporation. For in vivo inhibition, mice received injections i.v. daily with 20 mg/kg sCR1. To confirm C inhibition, mice were bled daily, and serum was tested for C hemolytic activity using rabbit erythrocytes (RbE) sensitized with mouse anti-RbE antiserum. Serial dilutions of test or control sera were incubated with 2% RbE. Hemolysis was measured by absorbance in supernatants at 415 nm ([A$_{415}$ (sample) − A$_{415}$ (min)]/[A$_{415}$ (max) − A$_{415}$ (min)] ×100). Hemolytic activity in samples taken 24 h posttreatment was always <15% of controls.

Results and Discussion

Induction of IL-2 production by anti-CD59 mAb

Previous cross-linking experiments indicate that human CD59 acts as an accessory molecule for T cell activation (3). To confirm this finding in the mouse system, we stimulated purified splenic CD4$^+$ T cells with PMA, CD59a-specific mAb and cross-linking anti-Ig. CD59a cross-linking on mouse CD4$^+$ T cells did not induce IL-2 production (Fig. 1A). Because this may be due to the low level of CD59a on mouse lymphocytes (as assessed by FACS; Ref. 9), we overexpressed CD59a in a
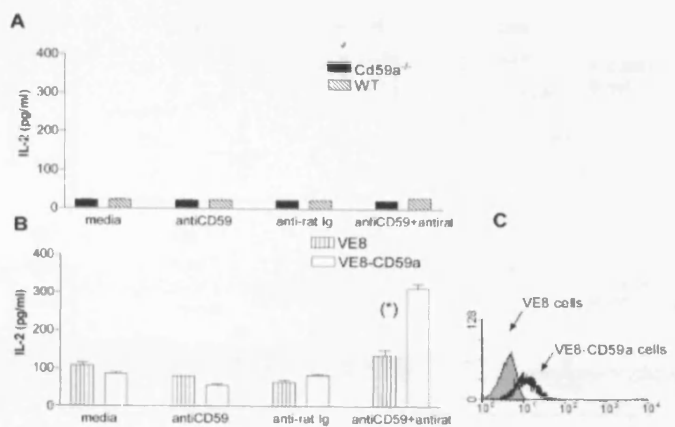
FIGURE 1. CD59a cross-linking induces secretion of IL-2. Expression of CD59a in VE8 and VE8-CD59a cells was detected by flow cytometry (C). Purified CD4$^+$ splenic T cells from WT and Cd59a$^{-/-}$ mice (A) or VE8 and VE8-CD59a cells (B) were incubated with mCD59.1 mAb followed by an anti-Ig Ab. After 18 h, IL-2 production was detected by ELISA. Values shown are mean ± SD. The results are representative of two experiments. Statistical significance (*) was evaluated using the Student's t test ($p < 0.001$).

murine CD4$^+$ T cell hybridoma (V8E) (6). Transfected cells expressed CD59a (Fig. 1C), and, when stimulated with PMA, cross-linking of CD59a yielded enhanced IL-2 production in comparison to untransfected cells subjected to the same stimuli (Fig. 1B). These data indicate that when CD59a is expressed at a sufficient level on murine T cells, cross-linking CD59a does enhance IL-2 production in a manner similar to that described for human T cells.

Mouse T cells express CD59a

CD59a expression is not detectable on murine lymphocytes by FACS (9). More sensitive methods were therefore used to determine whether primary murine CD4$^+$ and CD8$^+$ T cells express CD59a. CD59a expression was detectable by RT-PCR (data not shown) and immunoprecipitation, followed by Western blotting (Fig. 2A) in both CD4$^+$ and CD8$^+$ T cells from WT but not Cd59a$^{-/-}$ mice.

Immune responses in Cd59a$^{-/-}$ mice following infection with rVV

To determine whether physiological levels of CD59a plays any role in modulation of murine T cell activation in vivo, T cell responses were compared in WT and Cd59a$^{-/-}$ mice after rVVGP infection. rVVGP contains several MHC-restricted peptide epitopes recognized by T cells in WT mice. One peptide, gp33, derived from the gp, is presented by H2-D^b to CD8$^+$ T cells (14), whereas another epitope, p13, is presented by H2-I-A^b to CD4$^+$ T cells (12). No significant difference in CTL activity (Fig. 2B) or Ag-specific CD8$^+$ T cell proliferation (Fig. 2C) was observed between groups of mice at 8 days after infection. There was also no significant difference in CTL activity measured at day 42 after infection (data not shown). Virus-specific CD4$^+$ T cell proliferation assays performed at the same time points revealed stronger proliferative responses to the specific peptide, p13, in Cd59a$^{-/-}$ mice compared with WT mice, whereas no differences were observed in background proliferation to an irrelevant peptide, p61 (Figs. 2, D and E). These data are similar to those reported recently using CD55-deficient mice, where CD4$^+$ T lymphocytes from these mice were found
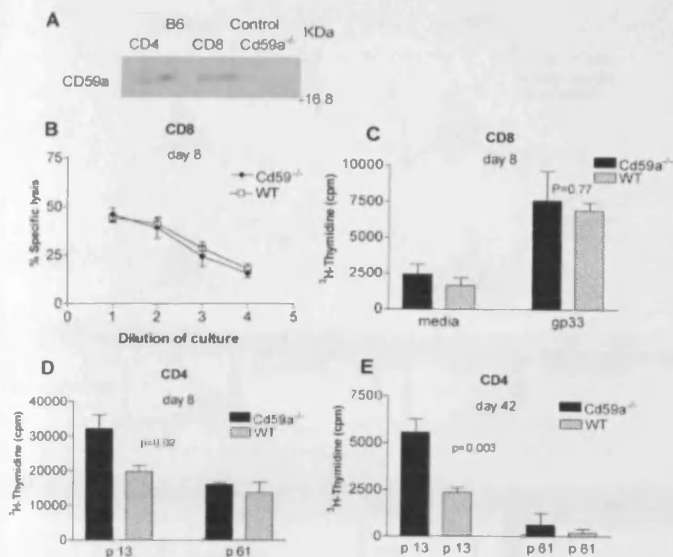
FIGURE 2. rVVGP-specific CD8⁺ T cell activity and CD4⁺ T cell proliferation. CD59a expression was analyzed from immunoprecipitates of purified CD4⁺ T and CD8⁺ T cells from WT and control T cells from Cd59a⁻/⁻ mice. Expression was detected using specific Abs in Western blotting (A). CD8⁺ T cell cytotoxic activity (B) and cell CD8⁺ T cell proliferation (C) was analyzed at day 8 after rVVGP infection. Nonspecific lysis in the experiment shown in (B) was <10%. CD4⁺ T cells purified from rVVGP-infected mice were tested for proliferation against peptides p61 and p13 8 days (D) and 6 wk (E) postinfection. Mice were analyzed individually, and values shown are the mean ± SEM (n = 3 mice/group). The results are representative of three independent experiments. Statistical significance was evaluated using the Student's t test.
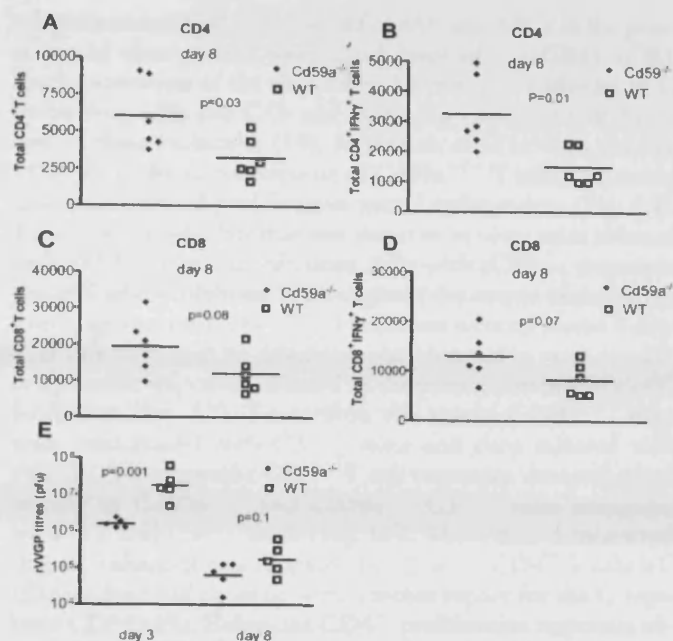

FIGURE 3. rVVGP clearance in Cd59a⁻/⁻ mice. Ovary-infiltrating lymphocytes were stained for intracellular IFN-γ, CD4, and CD8 expression at day 8 postinfection and analyzed by FACS. Total numbers of infiltrating CD4⁺ and CD8⁺ T cells are shown in A and C, respectively, and total numbers of IFN-γ producing CD4⁺ and CD8⁺ T cells are shown in B and D, respectively. Virus titers in the ovaries of infected mice were determined at day 3 and 8 postinfection (E). Each symbol represents an individual mouse, and similar data were observed in two independent experiments. Mean titers are also indicated in each graph. Statistical significance was evaluated using the Student's t test.

to proliferate more vigorously in response to a range of Ags compared with WT mice (15, 16). Another study reported that T cells isolated from Ly-6A-deficient animals also proliferate more vigorously than those isolated from their WT counterparts (17). Interestingly, Ly-6A, CD59, and CD55 are all GPI-anchored molecules localized in lipid rafts, and all have previously been found in cross-linking studies to promote T cell activation in vitro (3, 17, 18). Despite this, all three molecules negatively regulate T cell activity in vivo. Because GPI-linked proteins weakly associate with protein tyrosine kinases, it is possible that they can act either as positive or negative regulators of T cell activation by sequestering signaling molecules or interfering with assembly of signaling complexes in lipid rafts, the net effect dependent upon the trigger.

Immune responses in ovaries and virus titers

To determine whether the enhanced proliferation of gp-specific CD4⁺ T cells observed after in vitro stimulation reflected a stronger anti-viral CD4⁺ T cell response in vivo, numbers of CD4⁺ T cells at the site of rVVGP infection (ovary) were compared in WT and Cd59a⁻/⁻ mice at day 8 after infection. Higher numbers of infiltrating CD4⁺ T cells (Fig. 3A) and IFN-γ-producing CD4⁺ T cells (Fig. 3B) were observed in the ovaries of Cd59a⁻/⁻ mice compared with WT mice. The same analysis was performed for ovary-infiltrating CD8⁺ T cells (Figs. 3, C and D). Although higher numbers of CD8⁺ T cells were observed in the ovaries of Cd59a⁻/⁻ mice compared with WT mice, this difference was not statistically significant and may reflect enhanced helper CD4⁺ T cell activity rather than a direct effect of CD59a on CD8⁺ T activity. Virus titers were also compared in both groups of mice at days 3 and 8 postin-

fection. Virus titers were significantly lower in Cd59a⁻/⁻ mice compared with WT mice at day 3 postinfection, whereas both groups of mice had controlled the infection by day 8 (Fig. 3E). These data indicate that the more robust CD4⁺ T cell response observed in Cd59a⁻/⁻ mice does not reflect an inability to clear the virus efficiently but may rather contribute to more rapid control of the infection.

Analysis of CD4⁺ T cell proliferation in vitro

We next compared proliferation of T cells from WT mice and Cd59a⁻/⁻ mice after in vitro stimulation with CD3-specific mAb and APCs. Although no difference was observed in proliferation of CD8⁺ T cells (Fig. 4B), CD4⁺ T cells from Cd59a⁻/⁻ mice exhibited more proliferation in vitro compared with T cells from WT mice (2.5-fold increase; Fig. 4A). The increase in proliferation of CD4⁺ T cells from Cd59a⁻/⁻ mice compared with WT mice was only apparent when CD3-specific mAb and APCs were used for in vitro stimulation and not when CD3- and CD28-specific Abs were used (compare Figs. 4, B and C). To determine whether CD59a expression on APCs affected proliferation of the responding T cells, CD4⁺ T cells were incubated with APCs from WT or Cd59a⁻/⁻ mice. CD4⁺ T cell proliferation was not influenced by the presence or absence of CD59a on APCs, indicating that the difference in proliferation of CD4⁺ T cells from WT and Cd59a⁻/⁻ mice is due to expression of CD59a on the T cells (data shown). Incorporation of GPI-anchored CD59a into CD4⁺ T cells from Cd59a⁻/⁻ mice (Fig. 4D) caused a reduction in proliferation to levels the same as cells from WT mice (Fig. 4E). Together, these
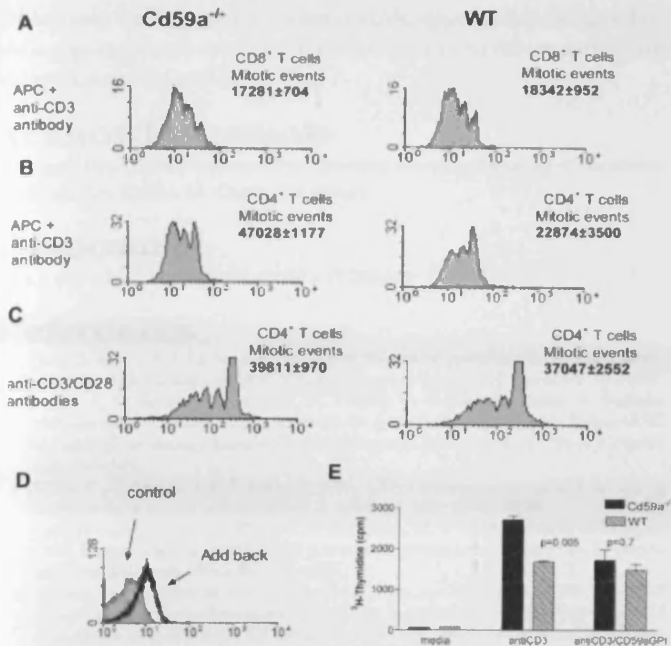
FIGURE 4. In vitro proliferation assays. Purified CD4$^+$ or CD8$^+$ T cells (>90%) were CFSE labeled and incubated with anti-CD3 mAb and APCs (*A* and *B*) or anti-CD28 mAb (*C*). Calculation of the number of mitotic events was performed as described previously (22). One representative result of at least three independent experiments is shown. Values indicating mitotic events are the means of three experiments ± SD. To confirm that lack of CD59a expression was responsible for enhanced proliferation of T cells from Cd59a$^{-/-}$ mice, lymphocytes were incubated with GPI-anchored CD59a (CD59a-GPI) before the proliferation assay (*E*). Incorporation of CD59a-GPI on CD4$^+$ T cells was confirmed by immunostaining (*D*). Values shown represent the mean ± SD. The experiment was performed on three separate occasions. Statistical analysis was performed by the Student's *t* test.

data imply that CD59a down-modulates CD4$^+$ T cell proliferation and requires the presence of APCs to exert this effect. It is possible that CD59a engages with a ligand on the APC or, alternatively, a soluble factor produced by the APC. To test the possibility that C activation and formation of the membrane attack complex mediates CD59a modulation of T cell activity, CD4$^+$ T cells purified from both WT and Cd59a$^{-/-}$ mice

were stimulated with CD3-specific mAb and APCs in the presence and absence of a soluble inhibitor of C (sCR1). sCR1 blocks activation of the classical and alternative pathways of C by binding C3b and C4b and mediating proteolytic degradation of these molecules (19). Inhibition of C in vitro did not affect the proliferative response of Cd59a$^{-/-}$ T cells, indicating that the enhanced proliferation was C independent (Fig. 5*A*). To determine whether this was also true in vivo, mice infected with rVVGP received injections daily with sCR1, a treatment that efficiently inhibited C throughout the course of the experiment. gp-specific CD4$^+$ T cell responses were measured 9 days after infection, and no difference was observed in virus-specific proliferative responses induced in the presence or absence of C inhibition (Fig. 5*B*). To confirm this result, Cd59a$^{-/-}$ mice were intercrossed with C3$^{-/-}$ mice and then infected with rVVGP. Virus-specific CD4$^+$ T cell responses were enhanced equally in Cd59a$^{-/-}$ and Cd59a$^{-/-}$C3$^{-/-}$ mice compared with WT and C3$^{-/-}$ mice (Fig. 5*C*). These data demonstrate that the enhanced proliferation of Cd59a$^{-/-}$ CD4$^+$ T cells is C independent and contrast with a recent report for the C regulator CD55 (15). Enhanced CD4$^+$ proliferative responses observed in CD55-deficient mice were largely, although not exclusively, C dependent.

In summary, this report identifies a role for the GPI-anchored C regulator CD59a in negative modulation of T cell activity in vivo. Due to the GPI anchor, CD59a is sequestered in membrane microdomains, which serve as platforms for Ag receptor signaling complexes in lymphocytes. CD59a may influence the rearrangement of lipid rafts after APC/CD4$^+$ T cell association. T cell activation induces a rapid compartmentalization of signaling machinery into lipid rafts. Costimulatory molecules are important for this redistribution (20), and it is possible that the presence of CD59a within rafts result in recruitment of different raft-associated kinases leading to modulation of T cell activity. No difference was found with CD8$^+$ cells, possibly due to differences in lipid raft composition between CD4$^+$ and CD8$^+$ cells (21).

Further analyses of Cd59a$^{-/-}$ mice are required to provide insight into the precise nature of the molecular events underlying the effect of CD59a on T cell activity. Such an understanding will reveal pathways through which CD59a and its human

FIGURE 5. Role of C in CD4$^+$ T cell proliferation. C was inhibited in vitro in assays where CD4$^+$ T cells were stimulated with CD3-specific mAb and APCs (*A*). Data are representative of two experiments, and values are shown as mean ± SD. C was inhibited in vivo by administration of sCR1 to mice for the first 9 days of infection with rVVGP (*B*). In vivo experiments were repeated with Cd59a$^{-/-}$ C3$^{-/-}$ mice (*C*). Mice were individually analyzed, and the values shown indicate the mean ± SEM (*n* = 3 mice/group). The results were analyzed statistically by the Student's *t* test.
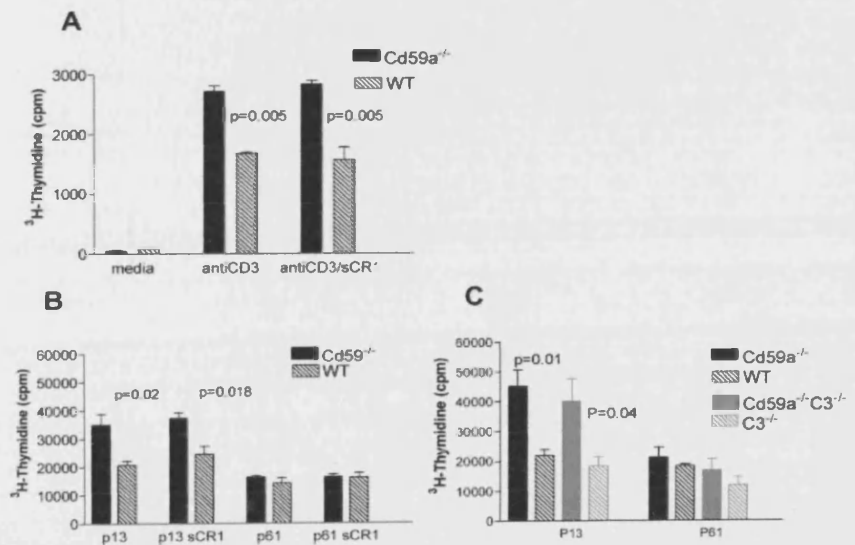
analog may be exploited for therapeutic approaches designed to either up-regulate beneficial T cell responses or down-modulate those that are harmful.

Acknowledgments

Disclosures

The authors have no financial conflict of interest.

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Holding T cells in check – a new role for complement regulators?

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Complement is not only part of the innate immune system, but has also been implicated in adaptive immunity. The role of complement and its regulatory proteins in modulating T cell activity has been the focus of several recent studies. These, which have included work on the membrane co-factor protein (MCP or CD46), decay accelerating factor (DAF or CD55) and CD59, indicate that complement regulators can influence the proliferative capacity of T cells and their ability to produce cytokines, influencing the outcome of a T cell response to a given antigen. Here we review these studies, which reveal another important link between the innate and the adaptive immune system.

Regulation of complement

Complement (C) has long been considered an arm of the innate immune system, as it is activated immediately in the presence of pathogen and provides a rapid and efficient means for opsonising or killing pathogens and triggering inflammation. As far back as the 1970s, however, there were indicators that C might have a role in adaptive immunity, particularly in the humoral immune response (reviewed in [1]). C activation is triggered via either the alternative pathway, which ticks over constantly in plasma providing an immediate means of defence against pathogens, or the classical pathway, triggered by binding of C1 to antibody or of mannan-binding lectin (MBL) to bacterial carbohydrate moieties (Box 1). Products of activation include the pro-inflammatory fragments C3a and C5a, which recruit leukocytes to sites of infection and activate these and other cells, and the opsonic fragments C3b and C4b, which label targets for elimination by phagocytes. All routes of activation culminate in the formation of a lytic transmembrane pore, the membrane attack complex (MAC). The C system is in a constant state of low-level tick-over activation, poised to amplify on targets and pepper adjacent innocent bystander cells with C fragments and complexes. To survive this potent threat, an armoury of regulatory proteins has evolved that protect self cells from damage by homologous C. Some of these C regulators (CRegs) are soluble, plasma proteins, whereas others are expressed on cell membranes; the latter group is the subject of this review.

The activation pathways are controlled by several membrane CRegs that belong to the 'regulators of C

activation' gene family and include decay accelerating factor (DAF or CD55) and membrane co-factor protein (MCP or CD46; reviewed in [2,3]). In common with all members of this family, they contain a structural module comprising ~60 amino acids, termed the short consensus repeat (SCR); the C3b-binding and functional activities of the proteins reside in this region [4]. CD46 and CD55 each contain four SCR modules (reviewed in [5]; Figure 1). On cell membranes, CD55 regulates C by binding to the C3 and C5 convertases, accelerating the decay of the enzyme subunits (decay accelerating activity) and thereby interfering with both the classical and alternative arms of the C cascade [(d) in Box 1] [6]. CD46 binds either C3b or C4b, freshly deposited or left on the membrane from decayed convertases, enabling a plasma serine protease, factor I (fI), to proteolytically cleave C3b or C4b (co-factor activity), preventing regeneration of the convertase (reviewed in [7]). In rodents, CD55 is broadly expressed and performs a similar CReg function [8,9]; by contrast, CD46 is absent from most murine tissues, expressed only in testis [10]. Instead, rodents have another broadly expressed membrane CReg, C receptor related protein-y (Crry) [11,12]. Crry is also built from SCR domains and performs the functions of both CD55 and CD46 [12,13].

Although several proteins collaborate to regulate the activation pathways of C, only one membrane CReg, CD59, inhibits the terminal pathway [14]. CD59, a small, globular glycoprotein (~20 kDa), is broadly expressed in tissues in humans and rodents and associated with the plasma membrane through a glycosyl phosphatidyl-inositol (GPI) anchor [15]. CD59 blocks formation of the lytic MAC by binding C8 within the C5b–8 complex, preventing unfolding and polymerisation of the final component, C9 [(d) in Box 1] [16]. Here, we review novel roles of these Cregs in modulating adaptive immune responses.

Modulation of T cell activation by CRegs
CD46 and Crry

Several recent publications and reviews have highlighted a role for CD46 in modulating T cell activity ([17] and reviewed in [18–20]). Simultaneous antibody ligation of CD3 and CD46 triggers induction of T cells showing characteristics of T regulatory 1-type cells (Tr1 cells). These cells secrete large amounts of interleukin-10 (IL-10) and inhibit proliferation of conventional CD4$^+$ T cells. Later studies revealed that CD46-induced Tr1 cells express high levels of granzyme B and perforin and can

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directly kill autologous targets [21]. Collectively, these observations suggest that Tr1 cells impinge upon other immune responses either through cell lysis or through immunosuppressive effects of IL-10. Importantly, these and other studies also showed that triggering with physiologically relevant ligand, such as C3b dimer or pathogen, induced the same phenotype [22,23].

As noted above, CD46 expression in rodents is restricted to testis and its C regulatory function is subsumed by the rodent-specific CReg, Crry. Like CD46, Crry might also influence T cell activation. Although studies using natural ligands to ligate Crry have not been performed, antibody crosslinking of Crry on murine T cells promotes T cell activation and the production of Th2 cytokines [24,25].

CD55 and CD59

There are two genes for CD59 in the mouse, encoding the widely distributed CD59a (the homologue of human CD59) and the testis-restricted CD59b [26,27]. Similarly, two genes for CD55 have been characterised in mice (named Daf1 and Daf2). Daf1 is GPI anchored, widely distributed and is the human homologue of CD55, whereas Daf2 is transmembrane anchored and is expressed only in testis and spleen [28,29]. Mice deficient in Daf1 or CD59a have been engineered and used to examine the influence of these proteins on T cell activity *in vitro* and *in vivo*.

Deficiency of Daf1 on T cells increases responses to antigen

Two recent studies describe T cell responses in CD55-deficient ($Daf1^{-/-}$) mice. The first study by Liu *et al.* [30] showed that recall responses measured using spleen cells from mice immunised with ovalbumin (OVA) or an MHC class II restricted myelin oligodendrocyte glycoprotein (MOG)-derived peptide were more vigorous in $Daf1^{-/-}$ mice than in controls. T cells from the $Daf1^{-/-}$ mice produced more interferon γ (IFNγ) and IL-2 upon antigen re-stimulation than their wild-type counterparts, whereas production of the immunosuppressive cytokine IL-10 was reduced. This hyper-responsiveness of T cells from the

$Daf1^{-/-}$ mice was due to lack of CD55 on the T cells themselves because antigen presenting cells (APCs) purified from $Daf1^{-/-}$ mice stimulated T cells to produce IFNγ to the same degree as those purified from $Daf1^{+/+}$ mice. The more vigorous response observed in $Daf1^{-/-}$ T cells was not due to a global increase in sensitivity of T cells to antigen stimulation because no difference was observed in the response of $Daf1^{-/-}$ and $Daf1^{+/+}$ T cells to stimulation with superantigen. A key finding was that the enhanced production of IL-2 and IFNγ by $Daf1^{-/-}$ T cells after re-stimulation with antigen was largely dependent upon a functional C system because the effect was lost when $Daf1^{-/-}$ mice were backcrossed onto C3-deficient ($C3^{-/-}$) mice in which C activity is abolished. A residual, C-independent effect of Daf1 deficiency on T cell function was suggested by the observation that T cell responses were more vigorous in $C3^{-/-}Daf1^{-/-}$ mice than in $C3^{-/-}Daf1^{+/+}$ mice. The C-independent effect was minor and unlikely to be of physiological relevance, as shown by studies in a T cell-driven experimental autoimmune encephalomyelitis model. Here disease was exacerbated in $Daf1^{-/-}$ mice, but not in $Daf1^{-/-}C3^{-/-}$ mice. Importantly, the increased T cell activity upon antigen re-stimulation *in vitro* was lost in $Daf1^{-/-}$ mice after neutralisation of C5 *in vivo*. This finding implies that C products downstream of C5, that is, the anaphylatoxin C5a or the MAC, are responsible for the observed phenotype in $Daf1^{-/-}$ mice.

Heeger *et al.* [31] independently reported similar findings in a different line of $Daf1^{-/-}$ mice. In this study, the authors observed that $Daf1^{-/-}$ spleen cells proliferated more vigorously following *in vitro* stimulation with allogeneic cells. In addition, more $Daf1^{-/-}$ spleen cells from female mice immunised with the male antigen (HY*Dby*) produced IFNγ upon restimulation *in vitro* than in controls. $Daf1^{-/-}$ female mice also rejected male skin grafts more efficiently than controls and had more HY-specific T cell activity in IFNγ ELISpot assays.

Although results described in these two studies are superficially similar and support a role for Daf1 in modulating T cell response, there are important differences. Liu *et al.* [30] reported that $Daf1^{-/-}$ and

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Box 1. C activation and regulation

C is activated via two pathways. The alternative pathway (Figure Ia, see next page) ticks over spontaneously in plasma (left), generating C3b-like molecules or C3b that can deposit on cell membranes. Membrane-bound C3b binds factor B (fB), which is cleaved and activated by factor D (fD) to form the C3 convertase (cleaving enzyme), C3bBb. This enzyme cleaves multiple molecules of C3 to C3b, which deposit on the membrane and form fresh C3 convertase enzymes or associate with the initiating enzyme to form C3bBbC3b, the C5 convertase. The C5 convertase cleaves multiple molecules of C5 to C5a and C5b, initiating the terminal pathway. The classical pathway (Figure Ib) is activated by antibody. The first component of the classical pathway, C1, binds antibody and becomes activated, leading to cleavage of C4 and deposition of C4b on the membrane. C2 associates with C4b and is cleaved and activated by C1 to form the C3 convertase, C4b2a. As with the alternative pathway, multiple molecules of C3b are cleaved and these associate with C4b2a to form the C5 convertase, C4b2a3b. The lectin pathway is identical to the classical pathway except that it is initiated by mannan-binding lectin (MBL) binding to carbohydrate moieties. Enzymes associated with MBL (MBL-associated serine proteases;

MASPs) then cleave C4 and C2 in a manner similar to C1. The activation pathways converge at the point of C5 cleavage (Figure Ic). C5b remains associated with the C5 convertase, where it binds C6 and C7. The C5b67 complex releases from the convertase and binds loosely to the membrane; it rapidly binds C8, forming the C5b678 complex. C9 binds to this complex and polymerises to form a lytic pore, termed the membrane attack complex (MAC). Regulation of the activation and terminal pathways is brought about through the actions of the membrane associated CReg: CD55, CD46 and CD59 (Figure Id). CD55 has decay accelerating activity and acts by binding the C3 and C5 convertases of all activation pathways (the alternative pathway C3 convertase is illustrated here). Binding of CD55 accelerates the decay of the enzyme by promoting the release of the enzyme subunit Bb or C2a. By dissociating the enzymes CD55 prevents amplification of the cascade by further C3 cleavage and enzyme formation. CD46 has cofactor activity: it binds C3b or C4b following decay of the convertase and enables a plasma serine protease, factor I (fI), to cleave and inactivate the protein. CD59 regulates the terminal pathway by binding the C5b678 complex, preventing correct C9 polymerisation and pore formation.
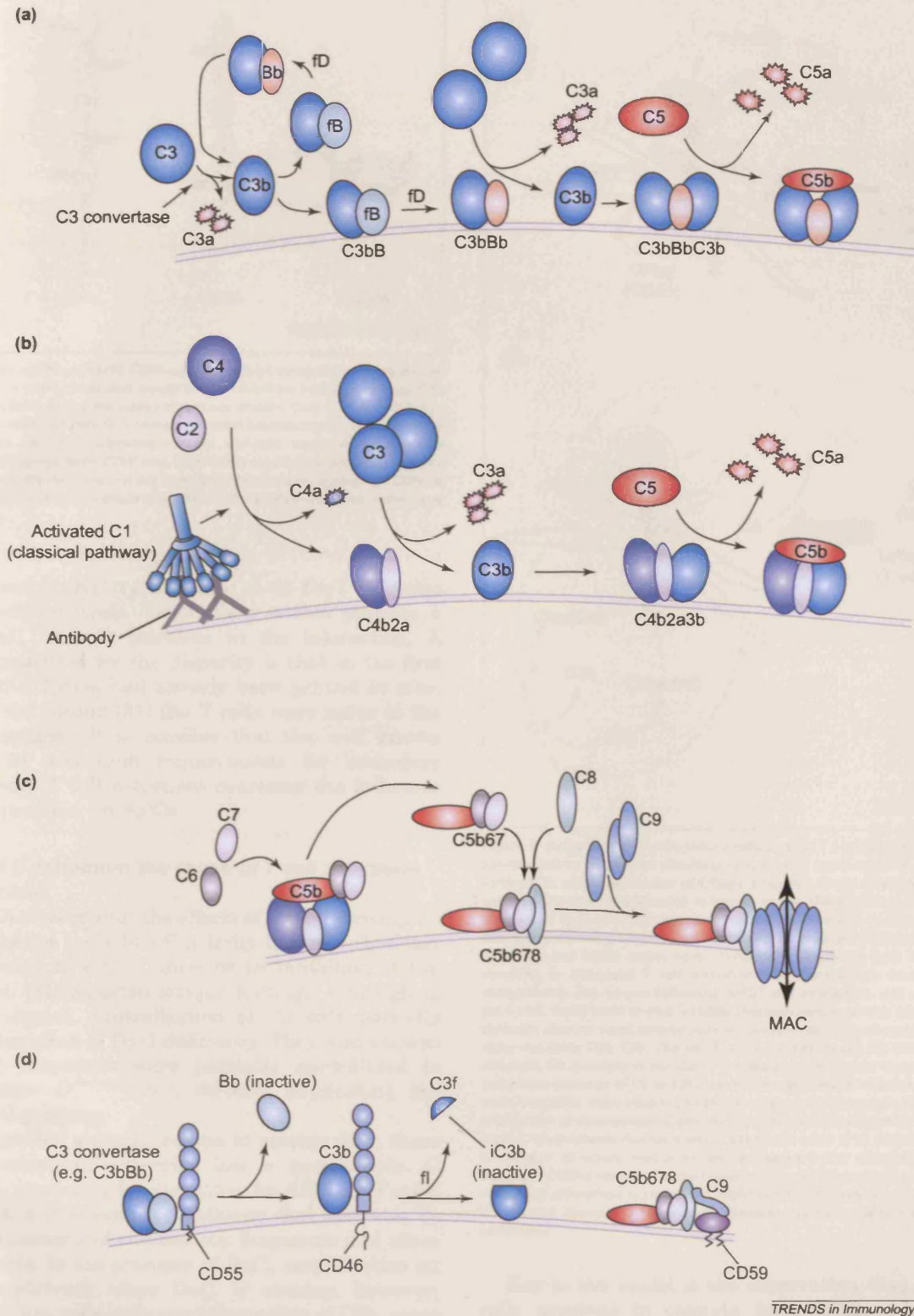
(Box 1) Figure I.

control APCs pulsed with OVA or MOG peptide stimulated T cells with equal efficiency and concluded that enhanced T cell proliferation in the $Daf1^{-/-}$ mice was exclusively a result of CD55 deficiency on the T

cells. By contrast, Heeger *et al.* [31] showed that the response of HY antigen-specific $Daf1^{+/+}$ T cells to $Daf1^{-/-}$ APCs was greater than that to control APCs. HY-specific transgenic T cells also proliferated
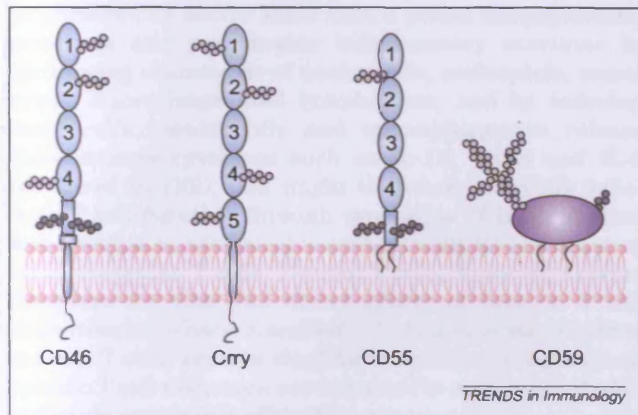
Figure 1. Structure of CRegs. CD46, CD55 and Crry are all composed of a structural module termed the short consensus repeat (SCR), which are linked 'end-to-end' in the molecule resulting in the elongated structures shown. Crry is only present in rodents and is formed from five SCR domains in mice (shown here) and six or seven SCR domains in rat. All molecules contain variable amounts of N-linked carbohydrate (light grey). Both CD55 and CD46 have membrane-proximal regions rich in serine/threonine residues that are heavily O-glycosylated (dark grey). CD59 is a small highly glycosylated molecule and, like CD55, is attached to the membrane via a GPI anchor.

more vigorously after transfer into male $Daf1^{-/-}$ mice compared with controls. These observations indicate a role for Daf1 on both partners in the interaction. A possible explanation for the disparity is that in the first study [30] the T cells had already been primed *in vivo*, whereas in the second [31] the T cells were naïve to the triggering antigen. It is possible that the well known differences in activation requirements for secondary versus primary T cell responses overcome the influence of CD55 expression on APCs.

Is increased C activation the cause of T cell activation in $Daf^{-/-}$ mice?

Liu *et al.* [30] showed that the effects of Daf1 deficiency on T cell activity are lost when C activity is removed, either by backcrossing to $C3^{-/-}$ mice or by inhibition of C5. Heeger *et al.* [31] reported similar findings, although in these experiments, neutralisation of C5 only partially abrogated the effect of Daf1 deficiency. They also showed that T cell responses were partially normalized in $Daf1^{-/-}Factor\ D^{-/-}$ mice, thereby implicating the alternative C pathway.

Taken together, a model begins to emerge from these studies in which C activation has a central role. C components, generated locally either by APCs or T cells, form a functional alternative pathway that activates C, generating opsonic and chemotactic fragments and other active products. In the presence of Daf1, amplification on T cells is restricted; when Daf1 is missing, however, amplification proceeds with more deposition of C3b, more stimulation by C5a and more triggering by MAC (Figure 2a,b). Although other CRegs such as Crry and CD59 are present on these cells, it is known that co-ordinated actions of multiple CRegs are required for efficient C regulation [32,33]; for example, deletion of CD59a in the mouse is sufficient to cause *in vivo* haemolysis [34].
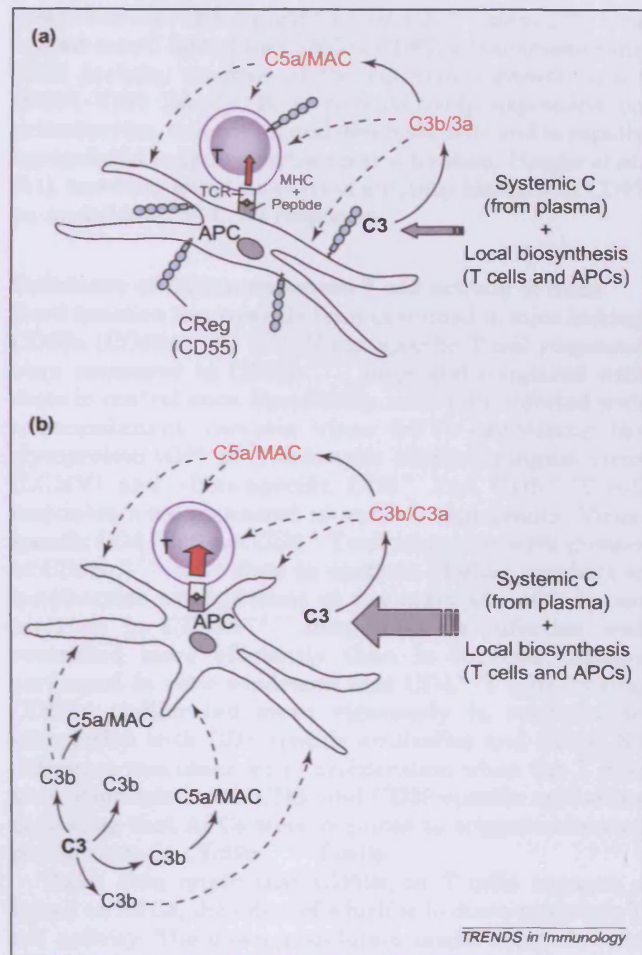
Figure 2. Interaction of C activation products with T cells and APC. (a) C ticks over spontaneously in plasma resulting in low level production of C3b. In wild-type animals, in which expression of CRegs is normal, levels of active complement are low and tick-over amplification is kept in check. The source of C is primarily from plasma. At sites of infection or inflammation (illustrated here) activation of the C cascade and production of active mediators is increased. These products (C3b, C3a, C5b, C5a and MAC) might have stimulatory effects on both APCs and T cells, resulting in enhanced T cell activation and proliferation (red arrow). Local C biosynthesis also occurs following APC/T cell interaction and contributes to the pool of C. Solid black arrows indicate cleavage events and/or progression of the C cascade; dashed black arrows indicate putative binding of activation products to receptors (only C3b, C3b, C5a and MAC are shown here). (b) When C regulation is deficient, for example in the $Daf1^{-/-}$ mouse, C tick over is more efficient, there is enhanced cleavage of C3 to C3b, particularly at sites of inflammation or infection, and C amplifies more readily on cell membranes. This results in C3b deposition and production of downstream C activation products. Following APC–T cell interaction, local C biosynthesis further contributes to the pool of C. Enhanced C activation, production of active mediators and subsequent cell stimulation occurs in the absence of CD55 resulting in the hyper-responsive phenotype seen in the $Daf1^{-/-}$ mice. This phenotype is absent in C-deficient (C3, fD) animals or can be reversed in C-sufficient animals by blocking activation products (which are in red) using antibodies.

Key to the model is the observation that APCs and T cells involved in cognate interactions synthesize the alternative pathway C components C3, fB and fD [31]. The model is analogous to that proposed for adipose tissue where local production of alternative pathway C components and local C activation is a physiological drive to lipid turnover [35]. The nature of the C activation products driving T cell activation is still a matter for conjecture. The observed inhibition by blocking C5

implicates C5a and/or MAC. C5a, a potent anaphylatoxin, promotes and perpetuates inflammatory reactions by facilitating chemotaxis of neutrophils, eosinophils, monocytes, macrophages and lymphocytes, and by inducing mast cells, neutrophils and macrophages to release inflammatory cytokines such as IL-12, TNFα and IL-6 (reviewed in [36]). C5a might therefore indirectly influence T cell function through promotion of inflammation and creation of a favourable environment for activation of antigen-specific T cells. Human T cells, however, express receptors for C5a and direct effects of C5a on T cell activation have been described [37]. It is not clear whether murine T cells express the C5a receptor, although virus-specific T cell responses are impaired in mice treated with a peptide antagonist of the C5a receptor [38].

MAC-induced cell activation has also been described in T cell lines. The relative contributions of C5a and MAC to the observed events should now be dissected using available inhibitors. Neutralisation of C5 did not completely abrogate the effect of Daf1 deficiency in some of the experiments described above, and it is possible that C3a and/or C3b might play a role (Figure 2a,b). C3a (as well as C5a) stimulates proliferation of hepatocytes and promotes regeneration of damaged liver [39]. As activated T cells express the C3a receptor, a similar mechanism might be employed for stimulation of lymphocytes [40]. Kerekes *et al.* [41] showed that deposition of C3b on APCs facilitates T cell proliferation through improving cell-to-cell contact between APC and cognate T cell. Although T cells express a variety of C3 receptors, including the C3b receptor, CR1, a systematic study of the effects of C fragments on T cell activity has not yet been performed (reviewed in [42]). Dissection of these effects will help elucidate the extent to which C and C-regulators modify T cell responses *in vivo*.

Also worthy of consideration is the possibility that a C-independent component might contribute to the hyperactivity of $Daf1^{-/-}$ T cells because C3-deficient $Daf1^{-/-}$ T cells showed enhanced responses to recall antigens compared with those purified from $C3^{-/-}$ mice [30]. One known non-C ligand for CD55 is CD97, a transmembrane (TM) protein, member of the epidermal growth factor (EGF)–TM7 family. It is constitutively expressed on granulocytes, monocytes and dendritic cells and is rapidly upregulated in lymphocytes upon activation. Heeger *et al.* [31], however, found no effect of antibody blocking of CD97 on modulation of T cell responses.

Deficiency of CD59a enhances T cell activity in mice

T cell function has recently been examined in mice lacking CD59a ($CD59a^{-/-}$) [43]. Virus specific T cell responses were measured in $CD59a^{-/-}$ mice and compared with those in control mice. Specifically, mice were infected with a recombinant vaccinia virus (rVV) expressing the glycoprotein (GP) of lymphocytic choriomeningitis virus (LCMV) and virus-specific CD4$^+$ and CD8$^+$ T cell responses were measured at various time points. Virus-specific CD4$^+$ but not CD8$^+$ T cell responses were greater in $CD59a^{-/-}$ mice than in controls. Higher numbers of lymphocytes were present at the main site of infection (ovaries) in $CD59a^{-/-}$ mice and the infection was controlled more efficiently than in controls. Assays performed *in vitro* confirmed that CD4$^+$ T cells lacking CD59a proliferated more vigorously in response to stimulation with CD3 specific antibodies and APCs. No difference was observed in proliferation when the T cells were stimulated with CD3- and CD28-specific antibodies indicating that APCs were required to trigger enhanced proliferation in $CD59a^{-/-}$ T cells.

These data imply that CD59a on T cells engages a ligand on APCs, the effect of which is to down-modulate T cell activity. The down-modulation might occur directly through inducing a negative signal in the T cell or transmitting a negative signal via effects on the APC (Figure 3a,b), or indirectly by interfering with the recruitment of Src family kinases during T cell activation thereby reducing the strength of the positive signal delivered to the T cells (Figure 3c). In marked contrast
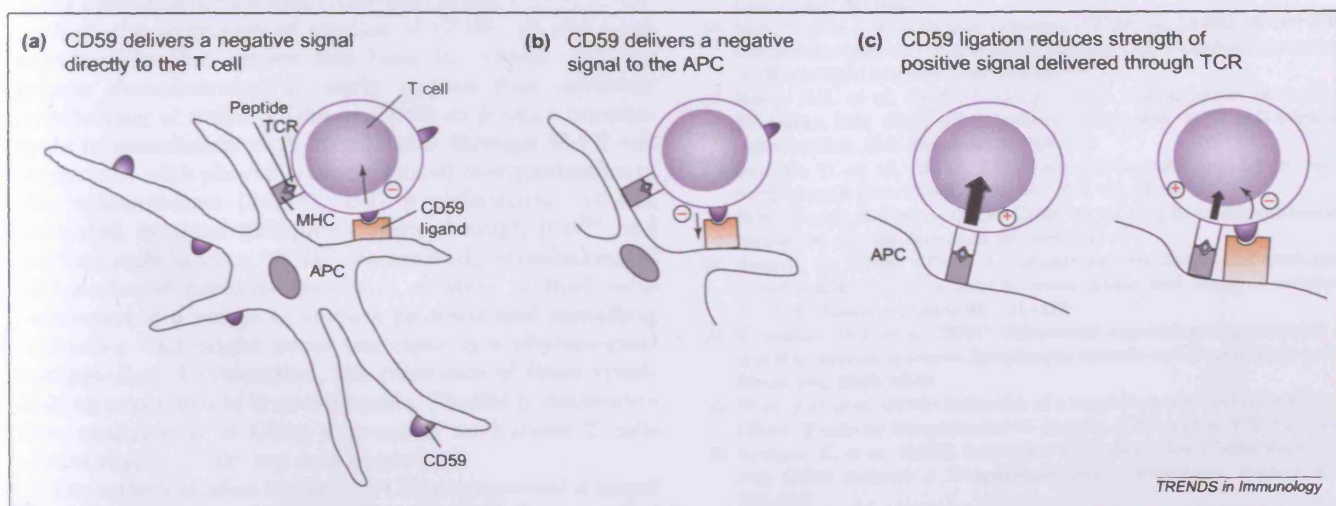
Figure 3. Effects of CD59 on T-cell–APC interactions. Direct interaction between CD59 (or CD55) on the T cell and a specific receptor on the APC results in an inhibitory signal being transmitted **(a)** to the T cell or **(b)** to the APC, resulting in down-modulation of APC activity and consequently T cell activity. **(c)** Alternatively, engagement of CD59 on T cells might reduce the strength of the positive signal delivered through the T cell receptor (TCR).

(a) CD59 delivers a negative signal directly to the T cell

(b) CD59 delivers a negative signal to the APC

(c) CD59 ligation reduces strength of positive signal delivered through TCR

T cell
Peptide
TCR
MHC
CD59 ligand
APC
CD59

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to the findings in *Daf1*$^{-/-}$ mice, the effect of CD59a deficiency on CD4$^+$ T cell proliferation was C independent. Neither inhibition of C using soluble complement receptor type 1 (sCR1) nor removal of C by backcrossing onto C3$^{-/-}$ mice reversed the effect of CD59a deficiency on CD4$^+$ T cell proliferation *in vitro* or *in vivo*. These experiments effectively eliminate the possibility that a C component ligates CD59a on T cells to mediate the down-modulatory effect.

Concluding remarks

The observations described here imply that CRegs, sometimes in collaboration with the C system, have key roles in regulating the adaptive immune system. It is notable that these CRegs, which serve to downregulate activation of the C cascade, also have the effect of downregulating T cell responses through several mechanisms. Engagement of CD46 by C component C3b results in induction of Tr1 cells that suppress the activity of other T cells and represents a mechanism through which C actively instructs the immune system to down-modulate antigen-specific responses. CD55 also holds T cell responses in check, although this occurs through limiting the stimulatory effects of C on both T cells and APCs rather than by altering the T cell phenotype. It is noteworthy, however, that antigen-stimulated T cells from Daf1$^{-/-}$ mice produced more of the immunosuppressive cytokine IL-10 than their wild-type counterparts.

Overall, it appears that under conditions of sustained C activation, such as tissue injury or chronic infection with a pathogen, CRegs serve not only to protect against the destructive effects of C but also to dampen down T cell response to avoid immunopathology and autoimmunity. It would now be informative to examine whether APCs and/or T cells modulate expression of CRegs according to their activation status as a means of fine-tuning their responses to C activation products. CD59, and perhaps also CD55, modulate T cell activation by processes independent of C activation. The discovery of CD59-binding ligands is urgently needed to aid the identification of the mechanism by which CD59 modulates T cell activity.

With the exception of studies of CD46, all the work described in this review has been in rodents. Several groups demonstrated in early papers that antibody crosslinking of either CD55 or CD59 on human lymphocytes in association with stimulation through the T cell receptor or with phorbol esters induced re-organization of the cytoskeleton [44,45], cell proliferation [46,47], enhanced tyrosine phosphorylation through p56^{lck} and calcium mobilisation [48,49]. As antibody crosslinking of GPI-anchored proteins generates clusters of lipid rafts sequestering a range of surface proteins and signalling molecules that might never associate in a physiological antigen–ligand interaction, the relevance of these crosslinking experiments is questionable. Studies to determine how modulation of CReg expression on human T cells affects their activity are now needed.

The rodent studies imply that CRegs represent a target for manipulation of T cell function for therapy. For example, tumour-specific T cells are subjected to a spectrum of inhibitory influences and much effort is being devoted to identifying ways to enhance the survival and proliferative capacity of anti-tumour T cell responses before re-infusion. If findings in mice are recapitulated in humans then the downregulation of CRegs would represent one such strategy.

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