The effect of two novel C-type lectins, Ba100 and Ba25, isolated from the venom of the puff adder, *Bitis arietans* on T lymphocyte proliferative responses

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DEDICATION

This thesis is dedicated to Jonathan
and my children, Rebecca, Laurence and Julian.

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- Finally, my family without whose support, encouragement and belief, none of this would have been achieved.

DECLARATION

This work is being submitted for the degree of Doctor of Philosophy (Medicine) at the University of Cape Town.

Neither the whole work nor any part of it has been, is being or is to be submitted for another degree in this or any other university.

The work described in this thesis was done in the Department of Medicine, Faculty of Health Sciences, University of Cape Town, under the supervision of Associate Professor Enid Shephard. The work is my own and where assistance from others has been received, their contribution has been acknowledged.

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ABSTRACT

The venom of the puff adder, *Bitis arietans*, is known to contain numerous bioactive components, including disintegrins and C-type lectins that modulate platelet function.

We have isolated two novel C-type lectins, which we called Ba100 and Ba25, using fibrinogen and platelet receptor affinity chromatography respectively, from the venom of the puff adder, *Bitis arietans* found in the Western Cape region of South Africa. Functional studies have demonstrated the ability of these two proteins to modulate platelet function either through their fibrinogenase activity (Ba100) or through their ability to target the platelet receptors GPIbα and $\alpha_2\beta_1$ (Ba25) (Jennings et al 1999, 2005 and unpublished data). As platelets are known to modulate intercellular interactions, cellular adhesion, transendothelial migration and share common receptors with lymphocytes, including the collagen receptor $\alpha_2\beta_1$, which is involved in T lymphocyte adhesion and signaling, we postulated that Ba100 and Ba25 might be able to modulate lymphocyte function.

The studies in this thesis investigated the effect of Ba100 and Ba25 on the proliferation of T lymphocytes in response to the mitogenic lectin Phytohaemagglutinin (PHA), several recall antigens and alloantigens participating in a mixed lymphocyte reaction. The results indicated quite clearly that both Ba100 and Ba25 could inhibit in a dose-dependent manner, calcium-dependent T lymphocyte proliferation mediated by these stimuli. Neither Ba100 nor Ba25 were able to inhibit calcium-independent T lymphocyte proliferation pathways such as proliferation in response to anti-CD28 antibody and a phorbol ester, phorbol 12-myristrate 13-acetate (PMA).

Proliferation in the presence of Ba100 at a concentration of 200 nM resulted in inhibition of the generation of lymphocytes expressing the activation markers HLA-DR and CD25 and downregulated the expression of adhesion/co-stimulatory molecules (CD11a, CD49d and CD29) and the costimulatory molecule CD28 on lymphocytes. Ba100 also inhibited the generation of T cell subsets that play a critical role in the alloimmune response: CTLs, NK cells and memory cells as well as increasing the CD4:CD8 ratio possibly reflecting the inability of CD8 lymphocytes to proliferate due to inhibition of the IL-2/IL-2 receptor pathway as Ba100 downregulated CD25 (IL-2 receptor α chain) expression.

Lymphocyte intracellular production of Th1 cytokines (IL-2, IFN-γ, TNF-α) and the Th2 cytokine IL-4 in response PMA and ionomycin stimulation in the presence of Ba100 (200nM) was inhibited. Investigation of lymphocyte progression through the cell cycle in response to PHA stimulation indicated that when Ba100 was included in the reaction, inhibition of cell cycle progression from the G0 – G1 phase into the S phase occurred. This was associated with downregulation of proliferating cell nuclear antigen (PCNA) expression.

Although the inclusion of Ba25 (200 nM) in PBMC proliferation reactions resulted in inhibition of proliferation, a minimal change in the expression of T cell activation markers was observed. An immunosuppressive cytokine profile characterized by increased IL-4 and IL-10 production and decreased IFN-γ production was detected during a mixed lymphocyte reaction in the presence of Ba25(200nM). Ba25 did not affect cell cycle progression nor proliferating cell nuclear antigen expression. The proliferation inhibitory properties of Ba25 appeared to be related rather to its ability to induce the generation of immunosuppressive prostaglandins. Indomethacin, a non-selective cyclooxygenase inhibitor added to PHA-induced PBMC proliferation reactions in the presence of Ba25 was able to reverse the Ba25-dependent inhibition of lymphocyte proliferation. We have not investigated the molecular mechanisms by which the C-type lectins Ba100 and Ba25 inhibit T lymphocyte proliferative responses. Possible mechanisms could include: i) acting as competitive ligands with lymphocyte integrin receptors, thereby preventing inside-out and outside-in lymphocyte signaling. This could be mediated through binding to the Lewis x trisaccharide which is known to bind other C-type lectins including the selectins, scavenger receptor C-type lectin and the dendritic cell receptor DC-SIGN; ii) interfering with optimal antigen-presenting cell function through competitive binding with their lectin receptors.

Thus, the ability of Ba100 and Ba25 to modulate platelet function and inhibit calcium-dependent T lymphocyte proliferation suggests a potential role for these two proteins in modulating the alloimmune response as both platelets and T lymphocytes are critical components of the inflammatory response. They may well prove after indepth analysis of their structure-function relationships to be capable of being developed as therapeutic agents that can prevent allograft rejection. Proof of concept rodent models of transplantation demonstrated that both Ba100 and Ba25 could prolong allograft survival without any histological evidence of haemorrhage, thrombosis or toxicity.

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CHAPTER ONE:

OVERVIEW

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1. **Introduction**

2. **Aim of the study**

CHAPTER ONE:

OVERVIEW

1. INTRODUCTION

The venoms of *Viperidae* and *Crotalidae* snakes are a rich source of proteins with biological activity against various factors involved in coagulation and fibrinolysis. They may act as procoagulants, anticoagulants or haemorrhagins (Ouyang et al 1992; Hutton & Warrel 1993; Marsh 1994; Markland 1998a; Lu et al 2005a). These properties have been utilized to analyse pathways of coagulation, to develop diagnostic assays assessing platelet function and coagulation as well as therapeutically in the development of anti-thrombotic agents (Marsh 2001; Morita 2004b; Marsh & Williams 2005; Schöni 2005; Wijeyewickrema et al 2005; Koh et al 2006). The bioactive components of snake venom proteins have been classified into various families including serine proteases, metalloproteinases, C-type lectins, disintegrins and phospholipases A(2) (Kamiguti et al 1998; Markland 1998; Morita 2004a). These bioactive components have been shown to target platelets, erythrocytes and leukocytes and C-type lectins are potent modulators of platelet function (Lu et al 2005b; Morita et al 2005; Ogawa et al 2005).

We have isolated two novel C-type lectins, Ba100 and Ba25, from the venom of the Western Cape, South African puff adder, *Bitis arietans*, using fibrinogen and platelet receptor affinity chromatography respectively and have demonstrated their ability to modulate platelet function (Jennings et al 1999; 2005). The venom of *Bitis arietans* is known to contain a disintegrin, Bitistatin (Shebuski et al 1989) and another C-type lectin, Bitiscetin (Hamako et al 1996), and both have platelet function modulating activity different from that of Ba100 and Ba25. Ba100 inhibited platelet aggregation in platelet-rich plasma and clot formation in whole blood. This was due to fibrinogenase activity of Ba100 that cleaves the fibrinogen Aα and Bβ chain preventing fibrinogen crosslinking and fibrin clot formation (Jennings et al 1999). Ba25 stimulated platelet agglutination in platelet-rich plasma and inhibited clot formation in whole blood. Ba25 interacts with the GPIbα receptor of the platelet von Willebrand receptor complex GPIb-IX-V close to the binding site of von Willebrand factor and the platelet collagen

receptor $\alpha_2\beta_1$ leading to activation of the platelet fibrinogen receptor GPIIaIIIb and increased fibrinogen binding. Ba25 also inhibited collagen-induced platelet P-selectin expression (Jennings et al 2005, Jennings unpublished data). Thus, these two novel C-type lectins modulate platelet function and prevent clot formation.

The C-type lectin protein family acting as recognition molecules also play a critical role in the immune system being involved in direct defense, cell recognition and trafficking within the immune system and in immune regulation. The C-type lectin-like domain (CTLD) family was originally classified into seven groups based on their overall domain structure (Weiss et al 1998; Drickamer 1999), but now consists of seventeen groups. The members of this CTLD superfamily and their diverse functions have been reviewed (Zelensky & Gready 2005). Very briefly, the C-type lectins playing an important role in the immune system are the:

i) Collectins including the serum mannose binding proteins and pulmonary surfactant proteins that are the first line of host defense recognizing pathogen-associated molecular patterns and initiating direct opsonisation, agglutination, complement activation and phagocytosis of pathogens (Van De Wetering et al 2004; Degn et al 2007; Gupta & Surolia 2007).

ii) Selectins (L-, E- and P-selectins) that direct adhesive interactions between leukocytes, platelets and the endothelium (Lasky 1992, 1995; Barthel et al 2007; Wang et al 2007). They play a critical role in the rolling of leukocytes along the endothelium and are involved in the initial recruitment of leukocytes into sites of inflammation or secondary lymphoid tissue (Kelly et al 2007). All 3 selectins recognize sulphated and sialylated derivatives of the Lewis x and Lewis a trisaccharide of the endothelium (Sanders et al 1996). Lymphocyte homing and recruitment is mediated by specific interaction between L-selectin and the carbohydrate ligand 6-sulpho sialyl Lewis x on high endothelial venules (Mitoma et al 2007).

iii) Dendritic cell receptors that are involved in antigen presentation and interactions with T lymphocytes: the dendritic cell inhibitory immunoreceptor, DCIR (Bates et al 1999), the dendritic cell lectin-DLEC (Arce et al 2001), DC-SIGN that internalizes antigen for presentation to T

cells (Engering et al 2002) and the dendritic cell associated lectins, Dectin 1 and 2 (Ariizumi et al 2000a,b; Yokota et al 2001; Kanazawa et al 2004). DC-SIGN, dectin-1 and dectin-2 act as pattern recognition receptors for pathogens (van Kooyk & Geijtenbeek 2002). Dectin-1 has been shown to promote T cell proliferation (Ariizumi 2000a).

iv) Scavenger receptor C-type lectin (SRCL), an endocytic endothelial receptor that binds to the leukocyte cell surface Lewis x trisaccharide (Nakamura et al 2001; Coombs et al 2005; Feinberg et al 2007).

v) The macrophage mannose receptor involved in direct defense, binding to and phagocytosing pathogens (East & Isacke 2002).

vi) The immunomodulatory natural killer lectin-like receptors: NKR-P1, Ly-49 and CD94/NKG2 that transduce both inhibitory and activating signals to the NK cell (Ryan et al 2001).

The ability of snake C-type lectins to modulate platelet function and haemostasis has been extensively investigated (Lu et al 2005b; Morita et al 2005; Ogawa et al 2005) and more recently, snake C-type lectin-like proteins have been shown to modulate integrin-mediated functions such as adhesion, migration and invasion of tumour cells (Sarray et al 2001, 2004, 2007). Although studies have shown that snake phospholipase A_2 and disintegrins are able to modulate neutrophil function affecting neutrophil migration, chemotaxis, apoptosis and superoxide production (Sundell et al 2003; Coelho et al 2004; Tseng et al 2004a,b; Gambero et al 2004), no research has been directed at investigating the ability of snake C-type lectin-like proteins to modulate T lymphocyte proliferative responses.

It is known that platelets play a major role in inflammation by releasing pro-inflammatory factors and expressing cell surface receptors that bind to other blood cells, the extracellular matrix and the endothelium (Kasirer-Friede et al 2007). Thus, platelets are able to modulate intercellular interactions, adhesion and transendothelial migration (Weyrich et al 2003). Studies have shown that platelets enhance CD4 lymphocyte adhesion to extracellular matrix under flow conditions by the formation of heterotypic platelet-lymphocyte co-aggregates involving the platelet receptor $\alpha IIb\beta_3$, $\beta 1$ integrins as well as the CD40 ligand and P-selectin glycoprotein ligand (PSGL-1) (Solpov et al

2006). Activated platelets promote activated T cell adhesion to fibronectin under flow via integrin ($\alpha_5\beta_1$ and $\alpha_L\beta_2$), CD40-CD40L and P-selectin-PSGL-1 mediated interactions (Shenkman et al 2006). Activated platelets have been shown to play a role in the alloimmune response and promote rejection by releasing the soluble form of CD154 which interacts with CD40 on the surface of antigen-presenting cells and dendritic cells leading to their activation (Xu et al 2006).

It has been shown that platelets in peripheral blood are significantly more activated in renal transplant recipients with acute rejection compared to patients with normal graft function (Zhang et al 2003). A clinical study in renal transplant patients with acute rejection demonstrated that Lipo-PGE1, a platelet activation inhibitor, shortened the graft function recovery time and improved both patient and graft 1 year survival times (Zhang et al 2005). Thus, platelet activation is an important component of the allograft rejection process.

Platelets have receptors that are common to lymphocytes ($\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha v\beta_3$ and $\alpha_6\beta_1$) and the endothelium ($\alpha_2\beta_1$, $\alpha v\beta 3$ and $\alpha_5\beta_1$) (Pribilia et al 2004, Kasirer-Friede et al 2007) and thus these 2 novel C-type lectins that have been shown to modulate platelet function, may modulate platelet-leukocyte, platelet-endothelial and leukocyte-leukocyte interactions by targeting common receptors.

Allograft rejection is a complex process involving receptors on host leukocytes, host platelets and the microvascular endothelial cells of the transplanted organ that promote cellular interactions resulting in localization, adhesion and concentration of leukocyte effector cells within the graft. Vascular endothelial cells provide a contiguous barrier separating tissue cells of vascularized organs from the molecular components of plasma and circulating blood cells (Gimbrone 1987). When vascularized organs are transplanted into an allogeneic host, endothelial cells of donor origin prevent host leukocytes from immediately attacking the allograft. With endothelial injury, the platelets adhere to the exposed extracellular matrix, the clotting cascade is activated, resulting in fibrin deposition which further promotes platelet aggregation and platelet-endothelial and platelet-leukocyte adhesive interactions. The endothelium, inflammatory leukocytes and platelets release pro-inflammatory factors including thromboxanes, leukotrienes, platelet activating factors and platelet-derived growth factors, thereby further promoting graft damage (Lemstrom et al 1995; Rocha et al

2003). Graft rejection is typically accompanied by evidence of localised intravascular coagulation with thrombus formation which is characterised by fibrin deposition in the rejected organ and infiltration of activated leukocytes (Orosz 1994).

Adhesion processes are a basic component underlying the immune response associated with graft rejection and thrombus formation. The specific adhesion of cells to other cells is mediated by selectins, integrin receptors and surface proteins of the immunoglobulin (Ig) superfamily (Smith 2008). Cell interactions with the extracellular matrix, comprising laminin, fibronectin, von Willebrand factor and thrombospondin occur via integrin receptor binding to the amino acid sequences Lys-Gly-Asp (KGD) (Kloczewiak et al 1984; Gresham et al 1992), Arg-Gly-Asp (RGD) (Ruoslahti & Pierschbacher 1987) and Leu-Asp-Val (LDV) found in these proteins (Ruoslahti 1996).

Host $CD4^+$ and $CD8^+$ T lymphocytes interacting with microvascular endothelial cells of the transplanted organ play a pivotal role in initiating the process of graft rejection. The recruitment of lymphocytes to sites of immune response or inflammation involves direct cell-cell adhesive interactions between lymphocytes and antigen-presenting vascular endothelial cells (Choi et al 2004; Kelly et al 2007; Rao et al 2007). This is mediated by various adhesion receptors including T lymphocyte receptors of the selectin (Lawrence & Springer 1991; Lasky 1992; McEver et al 1995) and Ig superfamily such as L-selectin and CD2 which bind endothelial cell carbohydrate moieties and LFA-3 of the Ig superfamily respectively. Adhesion is also mediated by lymphocyte VLA-4 ($\alpha_4\beta_1$) of the β_1 integrin family, and CD11a/CD18 of the β_2 integrin family which bind to extracellular matrix protein ligands and/or counter-receptors of the Ig superfamily, VCAM-1, ICAM-1 and ICAM-2 expressed on the surfaces of endothelial cells (Bevilacqua et al 1993; Pribilia et al 2004; Rao et al 2007; Smith 2008). ICAM-1 and ICAM-2, the endothelial ligands for LFA-1 (CD11a/CD18), are constitutively expressed, but VCAM-1, the ligand for VLA-4 is not expressed on unstimulated endothelium (Springer 1990a,b; Bevilacqua 1993; Kelly et al 2007). The expression and upregulation of the adhesive activity of these molecules is mediated by IL-1, IL-2, IL-4 and tumour necrosis factor (TNFα) and the initial interaction of lymphocyte CD2 receptors on memory T cells with LFA-3 on endothelial cells (Springer 1990a,b; Bevilacqua 1993).

Exposure of the endothelium to TNFα and IL-1, besides upregulating endothelial receptors such as ICAM-1, ICAM-2, VCAM-I and E-selectin (Pober et al 1986a,b; Nawroth & Stern 1986a, 1987a; Collins et al 1995a), also alters the surface of the endothelium from an anticoagulant state to a procoagulant state (Nawroth et al 1985; Nawroth & Stern 1986b, 1987b; Rao et al 2007). This is associated with changes in the synthesis and surface expression of endothelial proteins such as tissue factor (Nawroth et al 1985; Collins et al 1995b). Tissue factor plays a major role in promoting coagulation by activating factor XIa, followed by activation of factors IX, VIII, X and prothrombin and subsequently the assembly of the prothrombinase complex and thrombin generation (Nawroth et al 1985; Stern et al 1985). Thrombin, as a key enzyme in the hemostatic process, upregulates the expression of P-selectin on endothelial cells and platelets, initiates fibrin formation and activates the platelet $\alpha IIb\beta_3$ receptor which then binds dimeric fibrinogen molecules (Nawroth & Stern 1986b). Thrombus formation occurs as a result of platelets binding via β_3 integrin receptors and carbohydrate moieties to the fibrin meshwork and to endothelial cells expressing P-selectin and integrins of the Ig superfamily.

Thus, platelets and lymphocytes both play critical roles in allograft injury. Agents which are able to modulate the function of both platelets and lymphocytes inhibiting cell-cell and cell-extracellular matrix interactions as well as inhibiting the deposition of fibrin and subsequent thrombus formation could have the potential for inhibiting the thrombotic/vascular/immune responses associated with solid organ allograft rejection.

Hypothesis: The novel C-type lectins, Ba100 and Ba25, isolated from the venom of *Bitis arietans*, that have been shown to modulate platelet function could have the potential to modulate lymphocyte activation and the generation of effector mechanisms that are involved in the alloimmune response leading to allograft injury and thus could be developed as immunomodulatory agents.

2. AIM OF THE STUDY

The aim of the study was to assess the ability of the novel C-type lectins, Ba100 and Ba25, to inhibit T lymphocyte proliferative responses.

We investigated the ability of Ba100 and Ba25 to modulate:

1. Calcium-dependent T lymphocyte proliferation pathways:
    a. Peripheral blood mononuclear cell (PBMC) proliferation in response to the mitogenic lectin phytohaemagglutinin (PHA), recall antigens and allo-antigens.
    b. Isolated CD4 lymphocyte proliferation in response to OKT3 (a muromonoclonal anti-CD3 antibody) and fibronectin.
2. Calcium-independent T lymphocyte proliferation pathways:
    a. PBMC proliferation in response to anti-CD28 antibody and a phorbol ester, phorbol 12-myristate 13-acetate (PMA).
3. Lymphocyte populations and the phenotype of lymphocytes after PBMC proliferation in response to PHA or in a primary mixed lymphocyte reaction (MLR).
4. Suppressor cell activity and cytotoxic activity of MLR generated effectors.
5. Cytokine production.
6. Cell cycle progression and proliferating cell nuclear antigen expression.
7. Lymphocyte membrane stability.

8. Rodent models of transplantation.

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    b. Histological assessment following rat orthotopic renal transplantation

        - severity of rejection

        - histological evidence of toxicity

The data obtained from these studies indicating that Ba100 and Ba25 inhibited T lymphocyte proliferation processes has been compared with the known immunosuppressive agents Cyclosporin, FK506 (Tacrolimus) and Rapamycin that are routinely used in clinical practice as part of the immunosuppressive protocol to prevent allograft rejection.

CHAPTER TWO:

LITERATURE REVIEW : SNAKE VENOM – BIOACTIVE COMPONENTS, DIAGNOSTIC AND THERAPEUTIC USES

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CHAPTER TWO:

LITERATURE REVIEW : SNAKE VENOM – BIOACTIVE COMPONENTS, DIAGNOSTIC AND THERAPEUTIC USES

1. INTRODUCTION

Snake venom is a complex mixture of biologically active proteins and peptides that exert their toxicological effects by targeting their substrates or receptors following envenomation. The major toxicological effects include:

i) local tissue dying at the site of the snake bite
ii) flaccid paralysis
iii) myolysis
iv) haemorrhage and coagulopathy
v) renal failure
vi) cardiotoxicity

There are 5 major families of venomous snakes (Clemetson et al 2005; Du et al 2006)

i) *Hydrophiladae* family (mainly myotoxic)
ii) *Viperidae* and *Crotalidae* family (haemorrhagic)
iii) *Elapidae* family (neurotoxic and haemorrhagic)
iv) *Colubridae* family (toxic effects not characterized)

This review will concentrate on the toxic components of the Viperidae and Crotalidae family.

Snake toxins may have multiple targets and toxins from different snake venom protein families are able to bind to a common target (Clemetson et al 2001; Du et al 2006). The venom composition within snake species can show considerable geographic variation (Chippaux et al 1991). The underlying causes for this variation is not fully understood, but dietary variation in addition to genetics may be partly responsible for the variation in venom composition (Daltry et al 1996; Sasa et al 1999).

The venoms of the *Viperidae*, *Crotalidae* and *Elapidae* families affect haemostasis through their toxic effects on blood cells, plasma proteins and the blood vessel wall. These snake venom toxins include phospholipase A_2, serine proteases, metalloproteinases, disintegrins and C-type lectins (Ouyang et al 1992; Hutton & Warrel 1993; Marsh 1994; Markland 1998a; Clemetson et al 2005; Du et al 2006; Clemetson et al 2007).

Snake toxins such as metalloproteinases, serine proteases and C-type lectins are able to inhibit or activate a number of coagulation factors including fibrinogen, thrombin, prothrombin, factor V, factor IX and factor X. C-type lectins, disintegrins and disintegrin-like proteins are able to modulate platelet activity either directly or by using co-factors such as IgMκ (Navdaev et al 2001; Navdaev & Clemetson 2001) and von Willebrand factor (Brinkhous et al 1983; Hamako et al 1996).

Snake phospholipase A_2 causes haemolysis and the haemolytic activity is dependent on the charge of the phospholipase A_2 and its phospholipid substrate (Wang et al 1996; Zhao et al 1998; Doley et al 2004). Basic phospholipases are more haemolytic.
Galactose-specific C-type lectins, existing mainly as homodimers can agglutinate erythrocytes (Hirabayashi et al 1991; Ozeki et al 1994; Nikai et al 1995; Wang & Xu 2000).

Snake venom metalloproteinases, disintegrins and phospholipase A_2 can modulate leukocyte function (Du et al 2006). Metalloproteinases such as mocarhagin (*Naja mocambique*) cleave platelet GPIbα, but is also able to cleave a 10-amino acid peptide from the N-terminus of P-selectin glycoprotein ligand I (PSGL-I), preventing P-selectin binding and is able to disrupt platelet-neutrophil interactions (De Luca et al 1995a; Ward et al 1996; Du et al 2006).

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Triflamp, a metalloproteinase (*Trimeresurus flavoviridis*) reduces platelet-neutrophil adhesion through proteolysis of PSGL-1 (Tseng et al 2004a).

Disintegrins, by binding to integrins, are able to modulate leukocyte activation and their interactions with other cells. Jarastatin *(Bothrops jaracara)*, a RGD-containing disintegrin is able to induce integrin-coupled signaling, resulting in focal adhesion kinase and phosphoinositide-3-kinase activation in human neutrophils. Jarastatin induces Erk-2 translocation to the nucleus and delays spontaneous apoptosis of neutrophils (Coelho et al 2004). The disintegrin, Rhodostomin has been shown to block Mac-1 dependent adhesion of neutrophils to immobilized fibrinogen with a resultant decrease in superoxide production (Tseng et al 2004b).

Snake phospholipase A_2 (from the venom of *Naja mocambique*) causes neutrophil migration as a result of activation of endogenous phospholipase A_2, following activation of GTP-binding protein and protein kinase C (Gambero et al 2004; Du et al 2006).

Thus, snake venom components are able to target platelets, erythrocytes and leukocytes.

This chapter will concentrate on snake phospholipase A_2, metalloproteinases, disintegrins, snake C-type lectins and their bioactivity; the bioactivity of venom from *Bitis arietans* and briefly discuss the use of snake venom components in medicine.

2. SNAKE PHOSPHOLIPASE A_2

Snake phospholiase A_2 belongs to the enzyme superfamily that hydrolyses the sn-2 acyl groups of membrane phospholipids releasing arachidonic acid and lysophospholipids. Snake phospholipases may exist as monomers, homodimers and heterodimers and have variable enzyme activity. They may exert toxic effects on nerves and muscle, but are also toxic to platelets, leukocytes and erythrocytes.

Snake phospholipase A_2 can both induce or inhibit platelet activation, but the mechanisms are not yet defined and are not dependent on its pI value.

An acidic phospholipase A_2 from the venom of *Agkistrodon halys pallas* (Chen et al 1987), and praelongin 2bIII (*Acanthopsis praelongus*) (Sim 1998) inhibit platelet aggregation. An acidic PLA_2 (*Agkistrodon acutus*) inhibits ADP-induced platelet

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aggregation in platelet-rich plasma and induces aggregation of washed platelets (Chen & Chen 1989). The platelet aggregation effect could be inhibited by asprin suggesting that the generation of arachidonic acid from phospholipids in the platelet membrane and the formation of thromboxane may play a role in the induction of platelet aggregation (Chen & Chen 1989).

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3. SNAKE VENOM METALLOPROTEINASES (SVMPs)

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The SVMPs are a subgroup of the reprolysins. SVMPs are synthesized as latent precursor pro-proteinases which may undergo processing to produce the active proteinases and are classified according to structure into 4 main groups: i) MP-I has a single zinc-metalloproteinase, ii) MP-II contains MP-I and a disintegrin domain in the C-terminus, iii) MP-III contains MP-II and an additional cysteine-rich domain, iv) MP-IV has the MP-III domain structure with additional lectin-linked domains connected by disulphide bonds (Bjarnason & Fox 1995; Fox & Serrano 2005).

SVMPs affect haemostasis in various ways. They may be haemorrhagic, fibrinolytic, fibrinogenolytic and can inhibit platelet aggregation.

3.1. Fibrin(ogen)olytic activity of SVMPs

Several SVMPs as well as serine proteases with fibrinogenolytic and/or fibrinolytic activity have been identified (Mebs & Panholzer 1982; Takeya et al 1993; Marsh 1994; Omori-Satoh et al 1995; Yamakawa et al 1995; Jia et al 1996; Swenson & Markland 2005).

These fibrin(ogen)ases cleave either selectively or in combination the α and β chains of fibrin(ogen) and occasionally cleave the γ chain (Hung et al 1994; Hung & Chiou 1994; Assakura et al 1994).

These fibrin(ogen)olytic enzymes are direct acting and do not require other factors for their activity. The activity of these enzymes leads to either non-clottable fibrin(ogen) products or hypercoagulability if the protease has thrombin-like activity. The interaction of fibrinogen with its receptor, the integrin αIIbβ3 (GPIIbIIIa) on the platelet is

essential for haemostasis (Ruoslahti 1991) and is critical for mediating interactions between platelets in the early stages of thrombus formation. The enzymatic cleavage of soluble fibrinogen resulting in the formation of fibrinogen molecules that are unable to bind to αIIbβ3, interferes with platelet-platelet interactions that are important for aggregation.

Most SVMPs are both fibrinogenolytic and fibrinolytic and preferentially cleave the Aα-chain. The serine proteases preferentially cleave the Bβ-chain (Swenson & Markland 2005). Examples of SVMPs with both fibrinogenolytic and fibrinolytic activity include neuwiedase, an α-chain fibrinogenase from *Bothrops neuwiedii* (Rodrigues et al 2000, 2001; Izidoro et al 2003), fibrolase, an α-chain fibrinogenase from *Agkistrodon contortrix contortrix* (Ahmed et al 1990a, b) and brevelysin L6, an α-fibrinogenase from *Agkistrodon halys brevicaudus* (Terrada et al 1999).

Brevinase (*Agkistrodon blomhoffi brevicaudus*) is a serine proteinase with β-fibrinogenase and fibrinolytic activity (Lee et al 1999; Lee & Park 2000).

3.2. SVMPs targeting the platelet receptor

Some SVMPs have been found to target platelet receptors including GPIbα of the von Willebrand receptor GPIb-IX-V and the platelet collagen receptors, integrin $\alpha_2\beta_1$ and GPVI, thereby affecting platelet aggregation (Jia et al 1996).

Mocarhagin (*N. mocambique*) inhibits platelet aggregation by removing the fragment His-1-Glu-282 from GPIbα (Ward et al 1996). Crotalin (*Crotalus atrox*), a MP-I SVMP, inhibits platelet aggregation by the proteolysis of GPIbα (Wu et al 2001a). The MP-III SVMPs atrolysin A (*Crotalus atrox*) (Jia et al 1997) and jararhagin (*Bothrops jararaca*) (Paine et al 1992; De Luca et al 1995b) inhibit platelet adhesion to collagen through the collagen receptor $\alpha_2\beta_1$ and thereby prevent platelet activation. This is thought to be mediated through the cysteine-rich domain of the MP-III SVMPs. Alborhagin (*T. albolabris*) is able to activate platelets through the collagen receptor GPVI leading to GPIIbIIIa-dependent platelet aggregation (Andrews et al 2001).

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4. DISINTEGRINS

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Disintegrins are a family of cysteine-rich low molecular weight peptides (40-100 amino acids) that usually contain the RGD (Arg-Gly-Asp) sequence within an amino acid loop maintained by disulphide bridges and are able to bind to integrins. Disintegrins have been isolated from the venom of various *Viperidae* and *Crotalidae* snakes (Gould et al 1990; McLane et al 1998; Calvete et al 2005; Barja-Fidalgo et al 2005) and are formed either by the proteolytic processing of multidomain metalloproteinases (Kini & Evans 1992; Takeya et al 1993; Calvete et al 2005) or synthesized from short-coding mRNAs (Okuda et al 2002; Sanz et al 2006).

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Disintegrins can be divided into 5 different groups depending on the length and the number of disulphide bonds of the polypeptides (Gould et al 1990; Calvete et al 2000a, 2003, 2005).

i) Short disintegrins containing 41-51 amino acids and four disulphide bonds (echistatin, eristostatin, obtustatin).

ii) Medium sized disintegrins containing 70-73 amino acids and 6 disulphide bonds (barbourin, flavoridin, atrolysin E, trigramin, albolabrin, elegantin, agkistrostatin, applagin, batroxostatin).

iii) Long disintegrins containing a 84 amino acid polypeptide cross-linked by 7 disulphide bonds (bitistatin, bilitoxin-1).

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iv) PIII disintegrins are derived from PIII SVMPs (snake venom haemorrhagic metalloproteinases). They are modular proteins containing an N-terminal disintegrin-like domain of approximately 100 amino acids including 16 cysteine residues involved in the formation of eight disulphide bonds and a C-terminal 110-120 residue cysteine-rich domain cross-linked by six disulphide bonds (jararhagin, ecarin, trimerelysin, mocarhagin, graminelysin).

v) Dimeric disintegrins that include homo- and heterodimers. They contain subunits of about 67 amino acids with 10 cysteines involved in the formation of four intra-chain disulphide bonds and two interchain cysteine linkages (Calvete et al 2000b; Bilgrami et al 2004) (acostatin, piscivostatin, contortostatin, EMF-10, schistatin).

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More than one disintegrin may be found in the venom of the same viper, e.g. agkistrostatin and applagin are both found in the venom of *Agkistrodon piscivorus* (Chao et al 1989). In addition, more than one variant of a disintegrin may exist, such as the three variants of trigramin from the venom of *T. gramineus* (Dennis et al 1990). The venom of *Bitis arietans*, the sub-Saharan puff adder, contains five variants including bitistatin 1,2,3 & 4 (Shebuski et al 1989) and bitan α (Dennis et al 1990). It is not known whether these variants are as a result of multiple genes or as a result of population variation.

Some disintegrins have been shown to have homology with extracellular matrix proteins. A region of homology, containing the tetrapeptide sequence PRNP found in the α chain of fibrinogen in position 267-270 (Doolittle et al 1979) is found at the C terminus of trigramin, albolabrin and echistatin (Gould et al 1990). Homology has been shown between trigramin and the human von Willebrand factor precursor, collagen α_1 (I) and laminin B_1 (Huang et al 1989).

A number of high molecular weight protein ligands such as fibronectin, vitronectin, collagen, thrombospandin, laminin, fibrinogen, von Willebrand factor and complement C3bi contain RGD which acts as a common integrin recognition site (Ruoslahti & Pierschbacher 1987; Ruoslahti 1996).

Disintegrins bind with high affinity to the β_1 and β_3 families of integrins with a potency that is 500-2000 times greater than that of short RGDX peptides (Gould et al 1990; McLane et al 1998, 2004). The RGD sequence is the cell recognition site that enables the selective binding to integrins, thereby inhibiting integrin-related functions such as cellular adhesion and signal transduction (McLane et al 1998, 2004; Barja-Fidalgo et al 2005). Each disintegrin has a unique RGD containing integrin binding loop. The spatial configuration conferred by the disulphide bridges and the RGD integrin binding loop contribute to the higher binding affinity compared to linear RGD peptides (Gould et al 1990; Ruoslahti 1996; McLane et al 1998). The amino acid sequences adjacent to the RGD loop play a role in determining the biological activities and ligand-binding selectivity of disintegrins (Gould et al 1990; Ruoslahti 1996; Lu et al 1996; McLane et al 1998). Besides the RGD tripeptide, other tripeptide sequences such as KGD, MVD, MLD, VGD, ECD and MDG have also been identified as integrin-binding motifs in snake venom disintegrins (McLane et al 1998).

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Disintegrins not only inhibit integrin-related functions, but are also able to interact with and activate integrin-signaling pathways depending on the cell type and associated environment (McLane et al 2004; Barja-Fidalgo et al 2005). Disintegrins inhibit binding of RGD-containing ligands to the platelet receptor αIIbβ3 integrin, thereby impairing platelet aggregation responses dependent on this receptor (McLane et al 1994; Perutelli 1995). This binding of disintegrins to the αIIbβ3 integrin can occur without prior activation of this receptor. Interaction with the RGD-containing loop allows expression of the ligand-induced binding site (LIBS) on the β3 subunit (Juliano et al 1996; Kamiguti et al 1998). Disintegrins are potent inhibitors of fibrinogen binding to the glycoprotein GPIIbIIIa receptor of platelets (Huang et al 1987a, 1989; Gan et al 1988, Shebuski et al 1989, Chao et al 1989, Dennis et al 1990).

Jarastatin (JT) isolated from *Bothrops jararaca* venom has been shown to activate integrin-coupled signaling in neutrophils (Barja-Fidalgo et al 2005). JT activates MAPK pathway by inducing IL-8 synthesis, a potent inducer of migration, exocytosis and neutrophil respiratory burst (Baggiolini & Clark-Lewis 1992) and delays neutrophil apoptosis (Coelho et al 2004).

The monomeric disintegrins echistatin, kistrin and flavoridin are able to activate NF-kB via the PI3-K pathway, thereby interfering with CD25 expression and T lymphocyte proliferation (Barja-Fidalgo et al 2005).

Disintegrins have also been shown to interfere with other integrin-mediated cell functions (Kamiguti et al 1998) such as inhibition of tumour cell-extracellular matrix adhesion (Knudsen et al 1988; Sheu et al 1994; Beviglia et al 1995) and metastasis (Trikha et al 1994; Morris et al 1995).

The use of venom disintegrins has enabled the elucidation of cellular signaling events such as cellular activation and proliferation, cell adhesion and migration and programmed cell death that are mediated by adhesion molecules (McLane et al 2004; Barja-Fidalgo 2005).

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5. SNAKE C-TYPE LECTINS

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Snake C-type lectins are important haemorrhagic components of snake venom. They are named because of the resemblance of their prototypical foldings to those found in classic C-type lectins such as the selectins and the mannose binding protein (Chou et al 1996). The classic C-type lectins are calcium-dependent and carbohydrate binding. Most snake venom C-type lectin-like proteins do not bind carbohydrates and the calcium-binding loop is deleted.

The snake venom C-type lectins can be divided into the i) true C-type lectins with one canonical C-type lectin carbohydrate recognition domain (CRD) that binds sugars and agglutinates erythrocytes (Drickamer & Taylor 1993) and ii) the C-type lectin-like proteins with CRD-related non-carbohydrate binding C-type lectin-like domains (CTLDs) that do not bind sugars (Drickamer 1999; Clemetson et al 2005). The CRD-containing snake venom C-type lectins are disulphide-bond-linked homodimers and can form large multimers. The CTLD-containing snake venom C-type lectins are disulphide-bonded heterodimers composed of α and β subunits. These two subunits associate tightly and domain swopping between the subunits results in a conformational change in the central loop and formation of a concave surface (Lu et al 2005b; Ogawa et al 2005). The concave surface between the two subunits is thought to be the main binding site for the ligands, but has no carbohydrate binding ability. Both shape-fitting and electrostatic interactions play a role in binding (Lu et al 2005b). The snake C-type lectin-like proteins are divided into: i) coagulation-factor binding proteins binding to factors IX, X_a, thrombin and prothrombin or ii) platelet receptor binding proteins binding to the platelet receptors GPIb, GPVI, GPIaIIa ($\alpha_2\beta_1$) and CLEC-2 (Ogawa et al 2005; Morita et al 2005; Lu et al 2005b).

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These platelet receptor binding C-type lectin-like proteins may act as both platelet aggregation agonists and antagonists (Ogawa 2005). Only the interaction with platelet receptors and integrins will be discussed. See Table 1 for list of snake venom C-type lectins affecting haemostasis (Adapted from the list in the review paper by Lu et al 2005b).

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Table 1. Snake C-type lectins affecting haemostasis
(see the review paper by Q. Lu et al. Toxicon 2005b; 45: 1089-1098).

Protein name	Species	Target	Sequence
Mucetin	*Trimeresurus mucrosquamatus*	GPIb	+
Murocetin	*Trimeresurus mucrosquamatus*	GPIb	+
Flavocetin-A	*Trimeresurus flavoviridis*	GPIb	+
Mamushigin	*Agkistrodon halys blomhoffii*	GPIb	+
Alboaggregin-B	*Trimeresurus albolabris*	GPIb	-
Alboaggregin-C	*Trimeresurus albolabris*	GPIb	-
Agglucetin	*Agkistrodon acutus*	GPIb	+
GPIB-BP	*Bothrops jararaca*	GPIb	+
Agkicetin C	*Agkistrodon acutus*	GPIb	+
CHH-A and B	*Crotalus horridus horridus*	GPIb	+
Echicetin	*Echis carinatus*	GPIb, IgMκ	+
Lebecetin	*Macrovipera lebetina*	GPIb	-
TSV-GPIB-BP	*Trimeresurus stejnegeri*	GPIb	+
Tokaracetin	*Trimeresurus tokarensis*	GPIb	-
Purpureotin	*Trimeresurus purpureomaculatus*	GPIb	+
Convulxin	*Crotalus durissus terrificus*	GPVI, GPIb	+
Alboaggregin-A	*Trimeresurus albolabris*	GPIb, GPVI	+
Stejnulxin	*Trimeresurus stejnegeri*	GPVI	+
Ophioluxin	*Ophiophagus Hannah*	GPVI	-
Alboluxin	*Trimeresurus albolabris*	GPIb, GPVI	-
Botrocetin	*Bothrops jararaca*	VWF/GPIb	+
Bitiscetin	*Bitis arietans*	VWF/GPIb	+
EMS16	*Echis multisquamatus*	GPIa/IIa	+
Aggretin	*Calloselasma rhodostoma*	GPIb, GPIa/IIa	+
Rhodocetin	*Calloselasma rhodostoma*	GPIa/IIa	+
Bilinexin	*Agkistrodon bilineatus*	GPIb, GPIa/IIa	
Agkisacutacin	*Agkistrodon acutus*	Unknown	+
Agkaggregin	*Agkistrodon acutus*	Unknown	-
Rhodoaggretin	*Calloselasma rhodostoma*	Unknown	-
Jararaca IX/X-bp	*Bothrops jararaca*	IX/X	+
Habu IX/X bp	*Trimeresurus flavoviridis*	IX/X	+
TSV-FIX-BP	*Trimeresurus stejnegeri*	IX	+
AHP IX-bp	*Agkistrodon halys pallas*	IX	+
Halyxin	*Agkistrodon halys brevicaudus*	IX/X	+
ACF	*Agkistrodon acutus*	X	+
ECLV	*Echis carinatus leucogaster*	IX/X	+
Bothrojaracin	*Bothrops jararaca*	Thrombin	-
TSL	*Trimeresurus stejnegeri*	Galactose	+
LSL	*Lachesis stenophrys*	Galactose	+
RSL	*Crotalus atrox*	Galactose	+
BAL	*Bitis arietans*	Galactose	+
BjcuL	*Bothrops jararacussu*	Galactose	+

5.1. C-type lectin-like proteins that bind to platelet receptors

Platelets are critical in maintaining haemostasis both in an uninjured blood vessel and in limiting blood loss from an injured vessel. Important receptors involved in platelet aggregation include amongst others, the von Willebrand receptor complex GPIb-IX-V, the collagen receptors GPVI and the integrin $\alpha_2\beta_1$ as well as the platelet fibrinogen receptor, the integrin αIIbβ3 which also binds von Willebrand factor (vWF) (Ruggeri & Mendolicchio 2007; Kasirer-Friede et al 2007). The N-terminal 45 kDa domain of the GPIbα receptor contains the binding sites for vWF and α-thrombin (Du et al 2006).

Following injury to the blood vessel wall and exposure of vWF, platelets adhere to the site of injury and initiate formation of platelet plugs. Platelets adhere via GPIbα to the exposed A1 domain of vWF initiating platelet aggregation and thrombus formation (Du et al 2006). The platelet collagen receptor GPVI, an immunoglobulin family protein which is constitutively expressed, also plays a role in platelet aggregation by adhering to the exposed collagen (Kuijpers et al 2003; Lecut et al 2004).

Binding of vWF to GPIbα in the GPIb-IX-V receptor complex directly activates the collagen integrin receptor α2β1 increasing its affinity for collagen with subsequent activation of the αIIbβ3 receptor leading to platelet agglutination or aggregation depending on the level of signaling (Ozaki et al 2005; Du et al 2006). Direct collagen interaction with the platelet collagen receptors GPVI and α2β1 also activates αIIbβ3 (Lecut at al 2004). GPVI is associated with vWF receptor complex GPIb-IX-V on the membrane of resting and activated platelets. GPIb-IX-V and α2β1 play synergistic roles in platelet adhesion. Once adherent, the activated platelets release secondary agonists such as ADP, thromboxane A2 and serotonin and express adhesive protein ligands (Jin et al 1998; Turner et al 2001; Weyrich et al 2003; Kasirer-Friede et al 2007). The activated αIIbβ3 binds to fibrinogen which crosslinks the αIIbβ3 of platelets as well as binding to vWF leading to platelet aggregation. With the initiation of platelet aggregation, the coagulation cascade is also initiated leading prothrombin activation and thrombin formation. Thrombin further activates platelets by cleaving protease activated receptors expressed on the surface of platelets and propagates thrombus formation (Ruggeri & Mendolicchio 2007).

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These platelet adhesion receptors are targeted by several C-type lectin-like proteins and these proteins have played important roles in elucidating the structure/binding sites of platelet receptors as well as pathways of platelet activation (Wijeyewickrema et al 2005).

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5.1.1. GP1b-binding C-type lectin-like proteins

The snake C-type lectin-like proteins which act via GP1bα of the vWF binding complex either:

i) inhibit platelet activation by blocking binding of von Willebrand factor (vWF) and/or thrombin and these are usually simple heterodimers, examples of which are echicetin (*Echis carinatus*) (Peng et al 1993, 1994, 1995; Polgar et al 1997a), agkicetin (*Agkistrodon acutus*) (Chen & Tsai 1995) and lebecetin (*Macrovipera lebetina*) (Sarray et al 2003).

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ii) agglutinate or activate and aggregate platelets and these are usually multimeric and include the alboaggregins (*Trimeresurus albolabris*) (Andrews et al 1996; Kowalska et al 1998; Dormann et al 2001), agglucetin (*Agkistrodon acutus*) (Wang et al 2001), mucrocetin (*Trimeresurus mucrosquamatus*) (Huang et al 2004) and mucetin (*Trimeresurus mucrosquamatus*) (Lu et al 2004).

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Agglucetin (Wang et al 2001) and alboaggregin B (Peng et al 1991) agglutinates platelets via GP1bα without activating platelets. Alboaggregin A activates platelets via both GP1bα and the collagen receptor GPV1 (Dormann et al 2001). Mucetin and mucrocetin both activate platelets via GP1bα, but to different degrees. Mucetin binds to the platelet GP1bα, leading to αIIbβ3 activation and full platelet aggregation (Lu et al 2004) and mucrocetin only induces platelet agglutination (Huang et al 2004).

Agkicetin-C (*Deinagkistrodon acutus*) is a potent GPIb-IX-V antagonist and inhibits both vWF and thrombin interaction through binding to the AA201-282 region of GPIbα (Xu et al 2005).

The GP1b-binding proteins act differently in vitro and in vivo due to additional interactions with plasma and the endothelium.

5.1.2. von Willebrand factor (vWF)-binding C-type lectin-like proteins

Botrocetin and Bitiscetin are C-type lectin-like proteins that induce interactive vWF-GP1bα binding on platelets leading to agglutination and aggregation. The bitiscetin and botrocetin-induced binding depends on the optimization of interactions between the A1 domain of vWF and the 45-kDa domain of GP1bα with the C-type lectin-like proteins enhancing and stabilizing these interactions (Fukuda et al 2002; Maita et al 2003).

5.1.3. GPV1-binding C-type lectin-like proteins

GPV1 is the major signaling receptor for collagen on platelets and is a member of the Ig superfamily closely related to FcαR and NK receptors (Clemetson et al 1999). Crosslinking of GPV1 results in tyrosine phosphorylation of the ITAM (immunoreceptor tyrosine-based activation motif) of the Fcγ chain by the Src kinases Fyn and Lyn. This leads to binding and activation of the tandem SH2 domain-containing tyrosine kinase, Syk, initiating a downstream signaling cascade that activates several effector enzymes, including PLCγ2, small G-proteins and phosphoinositide-3 kinase. Clustering of GPV1 induces strong platelet activation. Convulxin (*Crotalus durissus terrificus*) (Polgar et al 1997b), alboaggregin A (*Trimeresurus albolabris*) (Asazuma et al 2001; Dormann et al 2001), stejnulxin (*Trimeresurus stejnegeri*) (Lee et al 2003) and ophioluxin (*Ophiophagus hannah*) (Du et al 2002a) are multimeric C-type lectins that induce platelet activation by clustering the receptor, GPV1. Ophioloxin and Stejnulxin are more GPVI specific than convulxin and alboaggrenin A which bind to other platelet receptors as well.

5.1.4. α2β1-binding C-type lectin-like proteins

The α2β1 integrin (GP1aIIa) is an important collagen receptor on platelets. α2β1 mediates adhesion and plays a role in generating intracellular signals needed to stabilize thrombus. Aggretin (*Calloselasma rhodostoma*) (Navdaev et al 2001b), rhodocetin (*Calloselasma rhodostoma*) (Eble & Tuckwell 2003), bilinexin (*Agkistrodon bilineatus*) (Du et al 2001) and EMS16 (*E. multisquamatus*) (Marcinkiewicz et al 2000) all interact with α2β1. EMS16 and Rhodocetin are potent and selective inhibitors of α2β1

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preventing platelet aggregation (Marcinkiewicz et al 2000; Eble & Tuckwell 2003). The action of Bilinexin and aggretin depends on their interaction with other platelet receptors in addition to α2β1.

5.1.5. CLEC-2 (C-type lectin receptor) binding C-type lectin-like proteins

Aggretin (rhodocytin) from the venom of *Calloselasma rhodostoma* has been shown to induce platelet activation through binding to GPIb-IX-V and the collagen receptor α2β1 and consequent tyrosine phosphorylation of Syk, p125 (FAK) and phospolipase Cγ2 without involvement of the PI3-kinase pathway (Navdaev et al 2001b; Chung et al 2001; Suzuki-Inoue et al 2001). Recent studies have shown that aggretin (rhodocytin) can induce platelet activation independent of these two receptors and GPVI, by binding of a novel C-type lectin receptor (CLEC-2) leading to tyrosine phosphorylation of its cytosolic tail, binding of Syk and initiation of downstream tyrosine phosphorylation events and activation of phospholipase Cγ2 (Bergmeier et al 2001; Suzuki-Inoue 2006).

5.1.6. GPIb, GPVI and GPIaIIa-binding C-type lectin-like proteins

Some snake C-type lectins may bind to more than one platelet receptor. Convulxin (*Crotalus durissus terrificus*) (Polgar et al 1997b; Kanaji et al 2003), Alboaggregin A (*Trimeresurus albolabris*) (Dormann et al 2001) and alboluxin (*Trimeresurus albolabris*) (Du et al 2002b) bind to both GPIbα and GPVI platelet receptors inducing platelet activation and aggregation. Aggretin (*Calloselasma rhodostoma*) (Navdaev et al 2001b) and bilinexin (*Agkistrodon bilineautus*) (Du et al 2001) bind to GPIb and the α2β1 (GPIaIIa) platelet receptors. Bilinexin agglutinates platelets and Aggretin strongly activates and aggregates platelets.

To date no snake C-type lectins binding to the platelet receptor αIIbβ3 have been identified.

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6. BITIS ARIETANS

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Using a combination of reversed phase HPLC and proteonomic analysis, the protein composition of the venom of puff adder, *Bitis arietans* has been found to consist of: Zn^{2+} -metalloproteinases (38.5%), serine proteases (19.5%), disintegrins (17.8%), C-type lectin-like proteins (13.2%), phospholipases A2 (4.3%), Kunitz-type inhibitors (4.1%), cystatins (1.7%) and unknown (0.9%) (Juárez et al 2006). Investigations into the potential diagnostic and therapeutic uses of the venom of *Bitis arietans* have focused on the disintegrin, Bitistatin and the C-type lectin, Bitiscetin.

6.1. Ba100

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We have isolated a novel protease, Ba100, which has similarity to C-type lectins, from the venom of the Western Cape, South African puff adder, *Bitis arietans*, using fibrinogen affinity chromatography (Jennings et al 1999). Ba100 has an apparent molecular mass of 100 kDa under non-reducing conditions and a pI of 5.4. Under reducing conditions, the protease dissociates into subunits of 21 kDa and 16 kDa. N-terminal amino acid sequencing has shown these two chains to have 66.7% homology as well as homology to the N-termini of other C-type lectins present in the venom from Crotalids and Vipers that are able to modulate platelet aggregation (Hamako et al 1996; Jennings et al 1999; Dormann et al 2001; Navdaev et al 2001b) (see Figure 1). The N-terminal sequence of the 16kDa subunit is PFCCPFGWSGYDQYCYKPFDEP and of the 21kDa subunit is DFECPTEW(C+S) (A+P)YDQHCYRAFDEP. However, the ability of Ba100 to bind to fibrinogen is thought to be independent of the carbohydrate binding domain present in C-type lectins as binding occurs in the absence of calcium or magnesium. The fibrinogenase activity of Ba100 initially cleaves the Aα and then the Bβ chain, but not the γ chain of fibrinogen, rendering the molecule unable to polymerise into fibrin clots. Reduced Ba100 is unable to degrade fibrinogen, suggesting that a disulphide-bonded structure may be required for enzyme function. Ba100 is distinct from previously characterised puff adder venom phospholipases, C-type lectin-like proteins, haemorrhaggins and another fibrinogenase with the ability to cleave the fibrin-specific γ chain crosslink site (Purves & Purves 1989; Júarez et al 2006). Both serine and metalloprotease inhibitors were able to partially inhibit the fibrinogenolytic activity

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of Ba100 suggesting that Ba100 probably has both serine protease and metalloproteinase activity.

Functional studies demonstrated the ability of Ba100 (25 nM - 1000 nM) to inhibit platelet aggregation in platelet-rich plasma, inhibit clot formation and reduce clot strength in whole blood in a concentration-dependent manner (Jennings et al 1999). The inhibition of the aggregation of platelets in platelet rich plasma appeared to be predominantly as a result of its proteolytic action on fibrinogen which prevents fibrinogen from crosslinking rather than through the binding of Ba100 to the platelet fibrinogen receptor GPIIbIIIa as no binding of Ba100 to purified GPIIbIIIa using affinity chromatography could be detected. The thromboelastogram findings that Ba100 prolonged the initiation of clot formation and reduced clot strength are in accordance with the fibrinogenolytic activity of Ba100 which interferes with the formation of a completely cross-linked fibrin matrix (Jennings et al 1999).

6.2. Ba25

Ba25, is another novel C-type lectin that we isolated from the venom of the Western Cape, South African puff adder, *Bitis arietans*, using a platelet receptor GPIIbIIIa affinity column. It was found to have an apparent molecular mass of 25 kDa under non-reducing conditions and a pI of 7.4. Ba25 is a heterodimer composed of disulphide-linked subunits of 18 kDa and 14 kDa and on amino-acid analysis was shown to be cysteine rich (Jennings et al 2005). N-terminal sequence analysis of the two subunits confirms that Ba25 is a novel protein and shows homology to another C-type lectin from *Bitis arietans*, Bitiscetin (Hamako et al 1996; Matsui et al 2002) and the fibrinogenase Ba100 (Jennings et al 1999). Ba25 also shows a high degree of homology to other C-type lectins in the venom from Vipers and Crotalids that modulate platelet aggregation (Peng et al 1994; Andrews et al 1996; Hamako et al 1996; Kawasaki et al 1996; Yamada et al 1996; Polgár et al 1997a; Kowalska et al 1998; Sakurai et al 1998; Sarray et al 2003). The N-terminal sequence of the 18kDa subunit is DQDCLSDWSSHEGHCYKVFDK and of the 14kDa subunit is ANCASGWAYGQHCYC (Jennings et al 2005) (see Figure 2).

Ba25 binds to the platelet fibrinogen receptor GPIIbIIIa isolated from intact platelets after immobilization on sepharose, thereby allowing large quantities of this protein to be

isolated devoid of other venom proteins (Jennings et al 2005). Our studies with intact platelets indicated that there may be little direct interaction of Ba25 with this receptor on intact live platelets in line with other studies with snake C-type lectins that have shown their lack of binding to GPIIbIIIa. FITC-labelled Ba25 did bind to intact resting platelets with increased binding when the platelets were stimulated with ADP. As ADP stimulation of platelets activates the platelet fibrinogen receptor GPIIbIIIa as well as the collagen receptor α2β1 (Kamae et al 2006; Schoenwaelder et al 2007), this suggested that Ba25 may associate with GPIIbIIIa on intact platelets, but may also interact with the collagen receptor α2β1. Flow cytometry analysis of the binding of specific antibodies anti-CD42b (clone SZ2) and anti-CD49b (clone Gi9) to platelet receptors GPIb and the α2 subunit of the collagen receptor α2β1 in the presence of Ba25 was used to elucidate potential platelet receptors to which Ba25 binds. These studies strongly suggested that Ba25 interacts with the GPIbα receptor of the GPIb-IX-V complex possibly close to the binding site of vWF, as well as interacting with the collagen receptor α2β1 (Jennings et al 2005 and Jennings unpublished data). Utilization of antibodies to fibrinogen binding sites on GPIIbIIIa allowed for the conclusion that the interaction of Ba25 with the GPIbα receptor induces activation of the platelet fibrinogen receptor GPIIbIIIa leading to increased fibrinogen binding and subsequently the observed platelet agglutination (Jennings et al 2005). Such interactions of Ba25 with GPIbα could be similar to that of the binding of vWF to GPIbα in the GPIb-IX-V complex which directly activates α2β1 to increase its affinity for collagen with subsequent activation of GPIIbIIIa and platelet agglutination or aggregation (Ozaki et al 2005; Du et al 2006). The ability of Ba25 to bind to multiple platelet receptors is a typical feature of C-type lectins (see section 5.1.6).

As aspirin did not inhibit the platelet aggregation capacity of Ba25, the metabolites of arachidonic acid are not involved in the aggregation process. Ba25 binding in the presence or absence of fibrinogen did not induce protein phosphorylation or the expression of procoagulant proteins on the platelet membrane (Jennings et al 2005 and Jennings unpublished data). Platelet aggregation by Ba25 in the presence of fibrinogen was not associated with α-granule release (no CD62P expression) or dense granule release, as measured by the release of radioactive serotonin (Jennings et al 2005). Ba25 binding to the GPIbα receptor and α2β1 probably induces a conformational change in

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the GPIIbIIIa receptor such that fibrinogen can bind in an activation independent manner with promotion of platelet agglutination. In addition, collagen-induced expression of P-selectin, the receptor involved in platelet binding to leukocytes, was prevented by Ba25 (Jennings et al 2005).

Bioactivity studies have shown that Ba25 stimulates platelet agglutination in platelet-rich plasma and promotes agglutination of washed platelets suspended in fibrinogen without the addition of other agglutinating stimuli. The thromboelastogram (TEG) demonstrated that Ba25 inhibited clot formation in whole blood by affecting all parameters of the TEG. In particular, the reduction in the reaction time suggested that Ba25 is an inhibitor of early stage blood clotting events such as cell-cell and cell-surface interactions. Ba25 had no inhibitory effect on aggregation of washed platelets in response to thrombin and no effect on clotting factors other than a possible involvement of the intrinsic pathway and von Willebrand factor.

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6.3. Bitistatin

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Bitistatin, a disintegrin with platelet aggregation inhibitory properties has been isolated from the venom of the sub-Saharan puff adder, *Bitis arietans* (Shebuski et al 1989). Bitistatin is a single-chain peptide containing 83 amino acids, 14 cysteine residues and is internally cross-linked with 7 disulphide bonds with a molecular mass of 9 kDa (Shebuski et al 1989; Gould et al 1990; Calvete et al 1997). Bitistatin contains the tripeptide sequence, RGD which is found in position 64-66 and binds with high affinity to the αIIbβ3 integrin (Knight & Romano 2005). The amino acid sequence of bitistatin shows considerable homology to other snake venom derived disintegrins: trigramin (68% homology) and echistatin (49% homology) (Shebuski et al 1989). Bitistatin was shown to inhibit ADP-induced human platelet aggregation in a dose dependent manner with the IC_{50} of 237 nM. The inhibitory effects of bitistatin on platelet aggregation are reversible. The bioactivity of bitistatin depends on its disulphide bonds as reduction and carboxyamidomethylation of the cysteine residues resulted in a 60-fold decrease in its inhibitory potency (Shebuski et al 1989).

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As Bitistatin binds to the platelet receptor GPIIbIIIa, a number of studies have investigated whether radiolabelled bitistatin could be used to diagnose acute peripheral

thrombi and pulmonary emboli. In animal models, the use of radiolabelled-Bitistatin has been able to diagnose deep vein thromboses and pulmonary embolism in a single test (Knight et al 1996; 2000). Short-chain Bitistatin analogues (Baidoo et al 2004) and a recombinant Bitistatin (Knight & Romano 2005) have also been developed for use in radiological imaging of thromboembolic disease.

In Phase I clinical studies, [99^{m}Tc]-r Bitistatin administered at low doses for imaging, binds to circulating platelets, but has no adverse effects on platelets and thus could potentially be used clinically to diagnose acute thrombo-embolic disease in humans (Knight et al 2007a).

Bitistatin has been successful in *in vivo* animal models in maintaining reperfusion and preventing reocclusion following experimental coronary artery thrombosis (Shebuski et al 1989; 1990) and in preventing platelet loss during extracorporeal circulation (Shigeta et al 1992).

The vitronectin receptor (αvβ3) is expressed on the surface of certain tumour cells, e.g. melanoma, breast cancer, glioblastoma and osteosarcoma. αvβ3 binds to ligands with the RGD sequence, modulating cell adhesion and migration and thereby influencing tumour angiogenesis and metastasis (Felding-Habermann & Cheresh 1993). αvβ3 is highly expressed on the endothelium of new vessels (Brooks et al 1994; Shattil et al 1995). Studies have investigated using radiolabelled Bitistatin as a diagnostic tool in malignancy. The use of radiolabelled Bitistatin (^{125}I-Bitistatin or ^{64}Cu-DOTA-Bitistatin) in microPET imaging confirmed tumour uptake and this was mediated by both αvβ3 and αIIbβ3 integrin binding (McQuade et al 2004). *In vitro* studies have shown that [99^{m}Tc]-bitistatin is internalized by tumour cells expressing αvβ3, but not by endothelial cells expressing αvβ3 (Knight et al 2007b). These properties of radiolabelled Bitistatin can potentially be used for the diagnosis and treatment of malignancy.

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6.4. Bitiscetin

Bitiscetin is a C-type lectin protein isolated from venom of *Bitis arietans*. Bitiscetin is a 25 kDa heterodimer composed of disulphide linked alpha (16kDa) and beta (13kDa) subunits with a pI of 9.1 (Hamako et al 1996).

Bitiscetin is a potent inducer of von Willebrand factor-dependent platelet agglutination. Bitiscetin activates the binding of the von Willebrand factor A1 domain to the platelet membrane glycoprotein Ib (GPIb). This activation is dependent on the formation of a bitiscetin-vWF A1 complex (Hirotsu et al 2001; Matsui et al 2002; Maita et al 2003; Matsui & Hamako 2005). The bitiscetin N-terminal sequence shows 71% homology with the 18kDa subunit of B25 and 52.6% homology with the 16 kDa subunit of Ba100 (see Figures 1 and 2).

The action of Bitiscetin is similar to that of Botrocetin (*B. jararaca*), a C-type lectin which also forms a stable complex with the A1 domain of vWF (Matsui et al 2002). Both botrocetin and bitiscetin activate vWF by binding close to the GPIb-binding surface, thereby inducing vWF-A1 binding to GPIbα without conformational changes in the vWF A1 domain (Hirotsu et al 2001; Sen et al 2001; Fukuda et al 2002; Maita et al 2003).

A distinct second form of Bitiscetin, Bitiscetin-2 has been isolated from the venom of *Bitis arietans* (Obert et al 1999, 2006). The 29 kDa Bitiscetin-2 is composed of 2 chains of similar molecular mass (± 15 kDa) and binds to the vWF A3 domain, inhibiting the binding of vWF to collagen (Obert et al 1999, 2006).

7. DIAGNOSTIC USES OF SNAKE VENOM

Snake venom toxins have been extensively utilized in the study of haemostasis and in assays for coagulation factors.

Snake venoms have varying effects on haemostasis (Marsh 1994; Markland 1997, 1998a; Pirkle 1998; Braud et al 2000; Matsui et al 2000; Lu et al 2005a). They may be coagulants (thrombin-like and prothrombin-activating toxins), anticoagulants (protein C

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activators), inhibitors/activators of platelet function, haemorrhagins and activators of fibrin(ogen)olysis.

7.1. Snake venom thrombin-like enzymes (SVTLEs)

SVTLEs include batroxobin and reptilase (*Bothrops atrox*), ancrod (*Callosellasma rhodostoma*) and ACTE (*Agkistrodon c. contortrix*). They are not inhibited by heparin and can be used to test plasma samples containing heparin or to remove fibrinogen from samples containing heparin (Marsh & Williams 2005).

The reptilase time can be used in place of the thrombin time as a rapid fibrinogen assay in samples containing heparin (Funk et al 1971). It can also be used to assay antithrombin III in plasma prepared free of fibrinogen (Howie et al 1973).

The reptilase time is prolonged in the presence of fibrin degradation products, hypofibrinogenaemia and in defects of fibrin polymerization (Latallo & Teisseyre 1971; Matsuda et al 1985; Cunningham et al 2002a). Very high fibrinogen titres may be associated with a prolonged reptilase time, but a normal thrombin time (van Cott et al 2002). Batroxobin (*Bothrops atrox*) can be used as a fibrin stimulant in a functional assay of tissue plasminogen activator (Ranby et al 1982).

7.2. Prothrombin activators

Commercially available prothrombin activators include ecarin (*Echis carinatus*), textarin (*Pseudonaja textilis*), Noscarin (*Notechis scutatus*) and an enzyme from the venom of Taipan (*Oxyuranus s. scutellatus*) (Rosing & Tans 1992). Snake venom prothrombin activators are of limited value in patients on warfarin as they cleave both normal prothrombin and functionally abnormal forms of prothrombin (Kornalik et al 1969). These prothrombin activators have been used to prepare non-enzymatic forms of meizothrombin and thrombin (Rosing & Tans 1992), to study prothrombin activation (Yamada et al 1996) and in the diagnosis of disseminated intravascular coagulation (Sakuragawa et al 1975) and dysprothrombinaemias (Weinger et al 1980; Collados et al 1997).

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The Pefakit APC-R factor V Leiden diagnostic kit includes the prothrombin activator noscarin and RVV-V, a factor V activator and is a sensitive screening test for factor V Leiden mutations (Wilmer et al 2004).

The Pefakit PICT (Prothrombinase induced clotting time) diagnostic assay which includes RVV-V was designed as a universal test to monitor the efficacy of anticoagulants such as low molecular heparin, unfractionated heparin, direct thrombin inhibitors and direct/indirect factor Xa inhibitors (Schöni 2005).

7.3. Factor V activators

A serine protease from the venom of Russell's viper is a selective activator of factor V (RVV-V) and has been used to assay factor V and its activation (Marsh & Williams 2005). It has also been used to define the cleavage sites in wild type and recombinant factor V (Keller et al 1995).

7.4. Factor X activators

Russell's viper venom also contains an activator of factor X (RVV-X) and has been used to assay factor X (Bachmann et al 1958), as a lupus anticoagulant assay (Thiagarajan et al 1986) and to distinguish between factor VII and factor X deficiency (Quick 1971). The Stypven time™ is the plasma clotting time using RVV-X (Denson 1961). A prolonged Stypven time suggests Factor X deficiency (Quick 1971). A normal Stypven time together with a prolonged prothrombin time suggests factor VII deficiency. RVV-X has also been used to identify Factor X recognition sites (Chattopadhyay & Fair 1989) and as an assay for platelet factor 3 (Jy et al 1995).

7.5. Assays for lupus anticoagulants

Lupus anticoagulants interfere with phospholipid-dependent clotting assays, i.e. activated partial thromboplastin time (APTT), prothrombin time (PT) and kaolin clotting time (KCT).

A number of snake venom activators have been used to assay lupus anticoagulant and include:

i) RVV-X (Thiagarajan et al 1986).

ii) Prothrombin activators from the venom of the taipan (*Oxyuranus scutellatus*), Australian brown snake (*Pseudonaja textilis*) and saw-scaled viper (*Echis carinatus*).

The dilute Russel's viper venom time (d RVVT) is a quick assay and variations of d RVVT has increased its sensitivity to assay lupus anticoagulant (Moore et al 2000).

A sensitive and relatively specific confirmatory test for lupus anticoagulant has been developed using Textarin™ from the Australian brown snake and Ecarin™ from the saw-scaled viper (Triplett et al 1993; Forastiero et al 1994). A Textarin/Ecarin ratio time of >1.3 is abnormal. A combination of the Taipan snake venom time and the Ecarin time was able to detect the presence of lupus anticoagulants in patients on Warfarin (Moore et al 2003).

7.6. Protein C activators

The active form of protein C (APC) plays an important role in haemostasis. It is a vitamin K-dependent anticoagulant proteinase that inactivates Factor Va and VIIIa. Protac, a protein C activator from the venom of the Southern copperhead snake (*A. contortrix contortrix*) (Stocker et al 1987) is used to measure activated protein C (Nathan et al 1987; Stocker et al 1988) and in functional clotting assays (Martinoli & Stocker 1986). A global clotting assay called ProC Global has been designed to assess the functionality of the protein C anticoagulant pathway. It is based on the ability of endogenous activated protein C generated by the activation of protein C by Protac to prolong the activated partial thromboplastin time. The ProcC global assay identifies carriers of Factor V Leiden, individuals with activated protein C resistance and protein C deficiency (Toulon et al 2000).

The ACV (*A. contortrix* venom) test based on the ratio of APTT in the absence or presence of Protac can be used to identify defects in the protein C anticoagulant pathway (Robert et al 1996).

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7.7. von Willebrand factor studies

Botrocetin (*Bothrops jararaca*) depends on von Willebrand factor to induce platelet aggregation and has been used to detect vWF activity (Read et al 1983; Brinkhous et al 1988). Botrocetin™ together with the antibiotic ristocetin have been used to detect molecular variants of vWF. Botrocetin partially aggregates platelets in patients with Bernard-Soulier disease where the glycoprotein Ib (GP1b) is absent, whereas ristocetin is unable to do this as it is dependent on GP1b.

The botrocetin/ristocetin combination has been used to characterize the human platelet vWF (Williams et al 1994). Alboaggregin B (*Trimesurus albolabris*), a vWF-dependent platelet aggregant has been used to quantitate vWF receptors on the GP1b molecule.

8. THERAPEUTIC USES OF SNAKE VENOMS

The bioactivity of various snake venom components has been used therapeutically in a wide variety of clinical conditions.

8.1. Neurotoxin

Cobrotoxin, a short-chain post-synaptic α-neurotoxin and α-cobrotoxin, a long chain α-neurotoxin, isolated from *Naja atra* have been shown to have analgesic activity in a rodent pain model. Cobrotoxin, a specific ligand for muscle-based α1 nAChR (nicotinic acetylcholine receptors) results in a centrally mediated analgesia via an opiate-independent mechanism. α-cobrotoxin has high affinity for the neuronal α7nAChR resulting in an opiate-independent, anti-nociceptive analgesic effect (Chen et al 2006; Zhang et al 2006).

8.2. Toxins affecting the cardiovascular system

Captopril, the first oral ACE inhibitor was developed following the observation that venom from *Bothrops jaracusa* caused severe hypotension and was found to contain an ACE inhibitor (Patlak 2003). Cardiotoxin from the Indian cobra has been used successfully in combination with crotoxin in the treatment of metastatic cancer (Costa et al 1998).

8.3. Phospholipase A_2 (PLA_2)

A PLA_2 isolated from *Naja Naja* venom is cytotoxic to Ehrlich ascites tumour cells and is a potential chemotherapeutic agent (Basavarajappa & Gowda 1992).

Crotoxin (*Crotalus durissus terrificus*), a cytotoxic PLA_2, is a pre-synaptic neurotoxin with cytotoxic activity and has shown promising results in a phase I clinical trial in patients with advanced cancer. There was documented reduction in primary tumour size and metastases (Cura et al 2002).

8.4. Toxins affecting haemostasis

These toxins are either activators or inhibitors of haemostasis and belong to the group of serine proteases, metalloproteinases, C-type lectins, disintegrins and phospholipases. These venom components have been used to develop inhibitors of platelet aggregation and blood clotting.

8.4.1. Thrombin-like enzymes

Thrombin cleaves both fibrinopeptide A and B from fibrinogen and activates Factor XIII. Snake venom thrombin-like enzymes can usually only cleave fibrinopeptide A, are unable to activate factor XIII and thus clots cannot be crosslinked and are easily broken down.

Thrombin-like enzymes which have clinically been used include Reptilase (*Bothrops atrax*) and Ancrod (*Callosellasma rhodostoma*). The STAT study (Stroke treatment with Ancrod Trial) showed that the administration of intravenous Ancrod compared to placebo was asscociated with a favourable 3 month functional status in patients with ischaemic strokes (Sherman et al 2000). Ancrod was less beneficial than the recombinant tissue type plasminogen activator or pro-urokinase (Fisher & Schaebitz 2000; Madhavan et al 2002). Ancrod has been used recently in the treatment of acute ischaemic stroke to limit infarct size (Samsa et al 2002). Ancrod has been used therapeutically in patients with heparin-associated thrombocytopaenia and thrombosis (HATT) syndrome (Illig & Ouriel 1996) and as an alternative to heparin in cardiopulmonary bypass (von Segesser et al 2001). Reptilase (Batroxobin) has been

shown to reduce the risk of stroke/transient ischaemic attacks in patients with concomitant hyperfibrinogenaemia (Xu et al 2007).

8.4.2. Prothrombin inhibitors

Defibrase (*Bothrops moojeni*) is a prothrombin/thrombin inhibitor and has been used in the treatment of angina pectoris and acute ischaemic strokes (Stocker & Barlow 1976).

8.4.3. Fibrin(ogen)olytic enzymes

The fibin(ogen)olytic enzymes are usually metalloproteinases or serine proteases. These enzymes break down fibrin-rich clots and prevent progression of clot formation. These properties have been used clinically to dissolve thrombus in intravascular catheters or for treatment of vascular thromboses. A number of fibrin(ogen)olytic enzymes have been tested in vitro and in vivo and include a fibrinogenase from *Vipera lebetina* (Gasmi et al 1997); afaacytin (*Cerastes cerastes*) (Laraba-Djebari et al 1995); fibrolase (*A. contortrix*) (Markland 1998b) and atroxase (Western diamond-back rattlesnake) (Baker & Tu 1996).

Alfimeprase, a truncated recombinant form of fibrolase has been used in phase II clinical trials for treatment of peripheral arterial occlusions and for management of occluded vascular access catheters (Toombs 2001; Swenson et al 2004).

8.4.4. Disintegrins

These integrin inhibitors have been used to design drugs to target and inhibit integrins, especially RGD-dependent integrins involved in disease processes, including thromboembolic disease, angiogenesis and malignancy. In thromboembolic disease, the aim is to target the platelet fibrinogen receptor αIIβ3. Drugs developed have included Eptifibatide (integrelin), a KGD-containing protein that was modeled on the active site of the disintegrin barbourin (*Sistrurus m. barbouri*) and Tirofiban (Aggrastat) that is modeled on the disintegrin echistatin (Scarborough et al 1991; Marwick 1998; Hantgan et al 2004). Eptifibatide and Tirofiban are both approved for the management of acute coronary ischaemic syndrome and prevention of thrombotic complications following coronary balloon angioplasty and coronary stenting (Pang et al 2002; Gilchrist 2003;

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van Werkum et al 2007; Zeymer & Wienbergen 2007; Casterella et al 2008; Marmur et al 2008).

Platelets are known to contribute to tumour growth, angiogenesis and metastasis (Trikha & Nakada 2002). Inhibitors of platelet integrins have been investigated as potential chemotherapeutic agents. The platelet receptor GPIIbIIIa complex interacts with vitronectin, fibrinogen, fibronectin and von Willebrand factor. The RGD-containing snake venom disintegrins interfere with platelet aggregation by reversibly blocking the GPIIbIIIa receptor. These disintegrins include trigramin (*Trimeresurus gramineus*), rhodostomin (*A. rhodostoma*) and triflavin (*T. flavoviridis*) (Huang et al 1987a, 1987b, 1991). The monomeric RGD disintegrins accutin (Yeh et al 1998), triflavin (Sheu et al 1997), rhodostomin (Yeh et al 2001; Huang et al 2001), salmosin (Kim et al 2004) and the homodimeric RGD-disintegrin contortrostatin (Zhou et al 2000) have been shown to inhibit angiogenesis by binding via αVβ3 to endothelial cells.

By blocking αVβ3 integrin in tumours, salmosin, triflavin and contortrostatin are able to inhibit the adhesion of tumour cells to ECM, reducing motility and inhibiting metastasis.

The fibronectin receptor, α5β1 integrin is a RGD-dependent integrin that plays a role in the pathology of vascular diseases (Hein et al 2001) and cancer (Adachi et al 2000) and is thus a potential therapeutic target using disintegrins.

MLD- and KTS-disintegrins are specific for leukocyte integrins and collagen receptors and are being investigated as potential therapeutic agents (Marcinkiewicz 2005).

8.4.5. C-type lectin-like proteins

Snake C-type lectin-like proteins have anti-coagulant, procoagulant and platelet-modulating activity. These properties can be used both therapeutically and diagnostically to elucidate pathways involved in haemostasis including platelet activation and clotting mechanisms as well as elucidating the structural-functional relationships of platelet glycoproteins and clotting factors (Morita 2004a,b, 2005; Clemetson et al 2007).

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Labectin and Labecetin, C-type lectins from venom of *Macrovipera lebetina* have been shown to bind to α5β1 and αV-containing integrins (Sarray et al 2004, 2007). Labectin has been shown both in vitro and in vivo to inhibit angiogenesis by blocking the adhesive properties of the fibronectin receptors, the α5β1 and αV integrins. This property could potentially be used to target and inhibit pathogenic angiogenesis (Pilorget et al 2007). Both Labectin and Labecetin have been shown in vitro to inhibit tumour cell adhesion, migration, invasion and proliferation by inhibiting α5β1 and αV-containing integrins (Sarray et al 2004, 2007) and this has potential therapeutic benefit in the management of malignancy.

Both convulxin (*Crotalus durissus terrificus*) and Crotacetin (*Crotalus durissus cascavella*) exhibit antimicrobial activity against Gram-positive and –negative bacteria (Rádis-Baptista et al 2006). The antimicrobial activity in convulxin is most likely also due to other contaminating proteins.

The diagnostic and therapeutic use of the bioactive components of snake venom has mainly focused on their ability to modulate haemostasis and very little research has been directed at their potential as immunomodulatory agents.

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Figure 1. Comparison of the Amino-terminal sequences of the 16kDa and the 21kDa subunits of Ba100 and other C-type lectins.

	Amino-terminal sequence																						% Sequence homology
p16	P	F	C	C	P	F	G	W	S	G	Y	D	Q	Y	C	Y	K	P	F	D	E	P	100
p21	D	F	E	C	P	T	E	W	S	P	Y	D	Q	H	C	Y	R	A	F	D	E	P	66.7
1	D	F	D	C	P	S	G	W	S	A	Y	D	W	Y	C	Y	K	P	F	N	E	P	76.2
2	G	F	C	C	P	L	G	W	S	S	Y	D	E	H	C	Y	Q	V	F	P	P	K	66.7
3	G	F	C	C	P	L	G	W	S	S	Y	D	E	H	C	Y	Q	V	F	Q	Q	K	60.0
4	G	A	D	C	P	S	G	W	S	S	Y	E	G	H	C	Y	K	P	F	N	E	P	68.4
5	D	F	N	C	P	P	G	W	S	A	Y	D	Q	Y	C	Y	Q	V	I	K	E	P	66.7
6			D	C	P	S	G	W	S	S	F	K	Q	Y	C	Y	K	P	F	K	Q	L	66.7
7	D	F	H	C	L	P	G	W	S	A	Y	D	Q	Y	C	Y	R	V	F	N	E	P	66.7
8	DP	G	C	L	P	-	G	W	S	S	Y	K	G	H	C	Y	K	V	F	K			52.6

Fig. 1. The 16 kDa and 21 kDa subunits of Ba100 show amino-terminal sequence homology with other C-type lectins from snake venom. Amino acids that are identical to those in the 16 kDa subunit of Ba100 are in blue. Sequence homology is shown as a percentage on the right. (1) Stejnaggregin-A from *Trimeresurus stejnegeri* (Chinese green tree viper); (2) Flavocetin-A from *Trimeresurus flavoviridis* (Habu); (3) Mucrocetin from *Trimeresurus mucrosquamatus* (Taiwan Habu); (4) Aggretin from *Agkistrodon rhodostoma* (Malayan pit viper); (5) Agglucetin-alpha from *Agkistrodon acutus* (Hundred pace snake); (6) Tokaracetin from *Protobothrops tokarensis* (Tokara habu); (7) Alboaggregin-A from *Trimeresurus albolabris* (White lipped pit viper), (8) Bitiscetin from *Bitis arietans*. Sequence data was obtained from http://www.ebi.ac.uk/fasta33/.

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Figure 2. Comparison of the Amino-terminal sequences of the 18kDa subunit of Ba25 and other C-type lectins.

	Amino-terminal sequence																					% Sequence homology
p18	D	Q	D	C	L	S	D	W	S	S	H	E	G	H	C	Y	K	V	F	D	K	100
1	D	Q	D	C	L	P	G	W	S	S	H	E	G	H	C	Y	K	V	F	N	L	85
2			D	C	L	P	G	W	S	S	H	E	G	H	C	Y	K	V	F	N	Q	79
3	A	A	D	C	P	S	D	W	S	S	H	E	G	H	C	Y	K	F	F	Q	Q	79
4	G	A	D	C	P	S	D	W	S	S	Y	E	G	H	C	Y	R	V	F	Q	K	79
5	G	A	D	C	L	S	G	W	S	S	Y	E	G	H	C	Y	K	A	F	E	K	79
6	D	P	G	C	L	P	D	W	S	S	Y	K	G	H	C	Y	K	V	F	K	K	71
7			D	C	P	S	D	W	S	S	Y	E	G	H	C	Y	R	V	F	N	E	74
8	D	Q	D	C	L	S	G	W	S	F	Y	E	G	H	C	Y	Q	L	F	R	L	74
9			D	C	P	P	D	W	S	S	Y	E	G	H	C	Y	R	F	F	K	E	70.6
10	G	A	D	C	P	S	G	W	S	S	Y	E	G	H	C	Y	K	P	F	N	E	68
11		A	N	C	A	S	G	W		A	Y	G	Q	H	C	Y	C					40
12	P	F	C	C	P	F	G	W	S	G	Y	D	Q	Y	C	Y	K	P	F	D	E	38

Fig. 2. Ba25 shows amino-terminal sequence homology with other C-type lectins from snake venom. Amino acids that are identical to those in the 18 kDa subunit of Ba25 are in blue. Sequence homology is shown as a percentage on the right. (1) Lebecetin from *Vipera lebetina*; (2) Carinactivase from *Echis carinatus*; (3) Bothrojaracin from *Bothrops jararaca*; (4) Mamushigin from *Agkistrodon halys blomhoffi*; (5) Factor IX/X binding protein from *Trimeresurus flavoviridis*; (6) Bitiscetin from *Bitis arietans*; (7) Alboaggregin from *Trimeresurus albolabris*; (8) Echicetin from *Echis carinatus*; (9) Botrocetin from *B. jararaca* (10) Aggretin from *Agkistrodon rhodostoma*; (11) The 18 kDa subunit of Ba25; (12) Ba100. Search conducted on UniProt database of EMBL-EBI (http://www.ebi.ac.uk/fasta33/).

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CHAPTER THREE:

LITERATURE REVIEW : MECHANISMS OF TRANSPLANT REJECTION

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CHAPTER THREE:

LITERATURE REVIEW : MECHANISMS OF TRANSPLANT REJECTION

1. INTRODUCTION

Allorejection is the ability of the T cells to recognise and respond to foreign histocompatibility antigens of other members of the same species. T cell recognition of the allo-antigen is the central event initiating cellular rejection of allografts.

The immune response to the transplanted organ can be divided into three phases: the afferent, efferent (effector) and regulatory phases. The ***afferent phase*** of the process involves activation of the recipient's immune system following recognition of foreign histocompatibility antigens (allo-antigens) of the organ donor by recipient lymphocytes. The initiation of this afferent phase occurs either in the lymphoid tissue of the recipient as a result of release of donor cells or allo-antigens from the graft or in the graft itself following infiltration by recipient lymphocytes into the transplanted organ.

The ***efferent phase*** occurs later as a result of triggering of both humoral and cellular effector mechanisms of the immune system by activated lymphocytes stimulated in the afferent phase of the response. The three main effector mechanisms include CD4 T cell responses especially the delayed-type hypersensitivity reaction; cytotoxic T cell responses and allo-antibody/complement responses (Rocha et al 2003). These combined cellular and humoral mechanisms lead to injury and the destruction of the transplanted organ. Thus, T lymphocytes and the major histocompatibility complexes are critical elements in the initiation of the rejection process.

Feedback regulatory mechanisms which downregulate activated T lymphocytes are essential for keeping immune responses in check and may contribute to allograft tolerance.

Cytokines and chemokines exert their actions in an autocrine or paracrine manner in order to orchestrate the different phases of the immune response (Abbas et al 1997).

2. CHARACTERISTICS OF THE ALLOIMMUNE RESPONSE

The principal histocompatibility antigens involved in the initiation of an allogeneic reaction are expressed on the plasma membranes of cells and are encoded by the major histocompatibility complex on chromosome 6 of humans (Krensky et al 1990, Krensky 1997). MHC class 1 molecules (HLA A, B, C) are expressed on all nucleated cells. MHC class II molecules (HLA DR, DQ, DP) are expressed on dendritic cells, macrophages, Kupffer cells and activated T and B cells (Unanue 1992). Crystallographic analysis of both HLA class I and II molecules showed a peptide antigen binding groove with an antiparallel beta-sheet floor and two sides composed of alpha helices (Brown et al 1993; Germain 1994; Jardetzky et al 1996). HLA polymorphisms allow binding of virtually infinite numbers of peptides determined by the interaction of specific antigen peptide motifs with anchoring residues in the antigen binding groove (Rudensky et al 1991; Hunt et al 1992a, 1992b). MHC class I molecules bind peptides from the cytoplasmic environment, which contain endogenous self peptides and viral peptides generated during viral infections, and present these peptides exclusively to CD8 T cells. MHC class II molecules bind exogenous peptides that have been endocytosed and processed within lysosomes and present exclusively to CD4 T cells. MHC also encodes other genes with immune function including transporters associated with antigen processing (TAP), large multifunctional proteosome components, tumour necrosis factors, complement proteins and heat shock protein 70. Recognition of foreign antigenic peptides in the context of MHC molecules by the T cell receptor, initiates T cell mediated immune responses (Krensky et al 1990; Krensky 1997).

2.1. Antigen processing, presentation and T cell receptor recognition: direct, indirect and semi-direct pathways

There are two distinct pathways of T cell allorecognition: the direct and indirect pathways. Transplanted organs express MHC molecules of the donor resulting in either direct or indirect antigen recognition (Sayegh et al 1994; Jiang et al 2004; Hornick 2006; Caballero et al 2006). The recipient T cell receptor (TCR) recognition of donor allogeneic-MHC is a principal event in the afferent phase of allograft rejection (Krensky et al 1990; Auchincloss & Sultan 1996; Magee & Sayegh 1997; Krensky 1997). TCR

allo-recognition involves interaction of the TCR/CD3 complex, the allo-MHC and the peptide antigen bound within the antigen-binding site of the MHC molecules. Thymic selection of T cells is based on the T cell recognition of peptide antigens expressed by self-MHC molecules (Suzuki et al 1995; Alam et al 1996; Marrack & Kappler 1997). Only TCRs with an intermediate affinity for self-MHC-antigen complexes which do not react strongly with self-antigens are positively selected. TCRs which do not react with self-MHC-antigen complexes, do not provide activation signals for survival and die. Those that react too strongly to self-MHC-antigen complexes are deleted. However, alloreactivity is the result of self-MHC-restricted TCR recognition of cross-reactive components of allogeneic MHC (Auchincloss & Sultan 1996; Magee & Sayegh 1997).

i) The direct pathway is characterised by the recognition of allo-MHC-peptide complexes on donor antigen presenting cells (APC) by recipient T cells (CD4 cells recognising allo-MHC class II and CD8 cells recognising allo-MHC class I). Dendritic cells are the alloantigen-presenting cells that mediate the priming of naïve T cells (Lechler & Batchelor 1982) and these dendritic cells are able to migrate to the recipient's lymphoid tissue (Larsen et al 1990). This pathway is responsible for the vigorous *in vitro* response seen in the primary allogeneic mixed lymphocyte reaction (MLR). *In vivo*, the direct pathway is the principal route of T cell sensitization leading to acute allograft rejection (Braun et al 1993). TCR stimulation in the direct pathway occurs because of molecular mimicry between the three-dimensional allogeneic MHC-peptide complexes and self-MHC-peptide complexes (Lechler et al 1990; Auchincloss & Sultan 1996; Magee & Sayegh 1997; Smith & Lutz 1997). Thus, even though the allo-MHC molecules are foreign proteins, they do not require processing and presentation as peptide antigens in order to stimulate the T cell response. This is confirmed in studies of reactions against allo-MHC molecules that do not contain endogenous peptide antigens in the antigen-binding sites and by the inhibition of alloreactivity with synthetic peptides mimicking the α-helices of the allo-MHC (Lechler & Lombardi 1991; Wecker & Auchincloss 1992). However, endogenous peptides, including processed MHC peptides, normally bound within the antigen-binding sites of the allo-MHC molecules, may also be involved in TCR stimulation via the direct pathway (Lombardi et al 1989; Mattson et al 1989; Lechler & Lombardi 1991). Thus, specific donor peptide antigens may also stimulate the alloreactive recipient's TCR. The direct pathway determines the strength of the allogeneic response as the precursor

frequency of T cells that directly recognise allo-MHC is high, 100 – 1000x greater than the response to nominal antigens (Lindahl & Wilson 1977; Sherman & Chattopadhyay 1993). Also, unlike the response to nominal antigens, the direct response to allo-MHC requires no previous exposure or priming and can be initiated by naïve T cells.

ii) The indirect pathway is characterised by recipient TCR stimulation by donor allo-antigens (allo-MHC or tissue-specific antigens) which have been processed and presented as peptides by recipient antigen-presenting cells. This pathway, thus reflects the normal physiological pathway of T cell recognition of foreign antigens or auto-antigens in the context of self MHC molecules as seen in infections and autoimmune diseases. The direct pathway is thought to be responsible for the initial vigorous immune response involved in acute rejection (Lechler & Batchelor 1982) with the indirect pathway playing a critical role in the development of chronic rejection (Vella et al 1997; Ciubotariu et al 1998a). The indirect pathway requires previous priming to antigen. The precursor frequency of T cells that recognise a specific antigen through the indirect pathway is relatively low, approximately 100 – 1000x lower than that for directly alloreactive T cells. The indirect pathway of allorecognition is able to induce the full range of alloresponses including proliferation and cytokine production by CD4 T cells (Benichou et al 1992); delayed type hypersensitivity responses (Waaga et al 1998); cytotoxic T lymphocyte generation (Lee et al 1994) and anti-donor IgG formation by B cells (Steele et al 1996).

There are two distinct features of the indirect pathway: ***immunodominance*** and ***epitope or antigen spreading.***

Immunodominance: In mouse and human models of transplantation, recipient MHC-restricted T cell responses were found to be limited to a single or a few dominant epitopes on the donor MHC antigen (Liu et al 1993; Benichou et al 1994). These immunodominant epitopes represent allogeneic determinants that are efficiently processed and presented by host APCs to activate T cells. Immunodominance reflects a hierarchy of allogeneic determinants that are recognised by T cells via the indirect pathway. During graft rejection, the recipient T cells are sensitised to a limited number of allogeneic determinants, despite the presence of numerous other foreign determinants (Sercarz et al 1993).

Epitope or antigen spreading: The hierarchy of allogeneic determinants of donor MHC peptides that are recognised by the indirect pathway can change during the course of rejection. Early in the course of rejection, T cell responses are limited to a single or a few immunodominant determinants. With time, the alloresponse can shift to another allodeterminant on the same (intramolecular) or different MHC molecule (intermolecular spreading) (Benichou et al 1992, 1994). This spreading of antigenic determinants may reflect differences in antigen processing from various anatomic sites or differences in T cell trafficking during the rejection process. Epitope spreading may be important in the amplification and progression of allograft rejection as the emergence of different allogeneic determinants leads to the recruitment of different T cell clones over time. The occurrence of epitope spreading was shown to correlate with the frequency of chronic rejection in heart transplant patients (Ciubotariu et al 1998a).

T cells that are primed to intact allo-MHC through the direct pathway are present in high frequency and induce CTL formation, delayed hypersensitivity reactions and allo-antibody formation leading to acute cellular rejection. The importance of the direct pathway decreases over time with the depletion of donor passenger leukocytes and the importance of the indirect pathway increases as T cells begin to recognise new epitopes via epitope spreading. The indirectly primed T cells produce cytokines, chemokines, allo-antibodies, growth factors (PDGF, FGF, TGF-β), activate macrophages and provide help for B cells and precursor CTLs. The walls of intraparenchymal graft vessels are targeted and transplant vasculopathy, the hallmark of chronic rejection, results.

iii) ***The semi-direct pathway*** of allorecognition has recently been proposed linking the direct and indirect pathways (Caballero et al 2006). Intact donor MHC molecules are transferred from donor to recipient antigen-presenting cells and then presented to recipient T cells. Recipient DCs acquire and present donor MHC class I molecules to direct pathway CD8 T cells and internalized and processed donor MHC class II molecules are presented as peptides to CD4 T cells with indirect allospecificity (Caballero et al 2006).

2.2. T cell activation in the alloimmune response

As described above, the alloimmune response involves the interaction of both naïve and memory alloreactive T cells with antigen-presenting cells (dendritic cells) of both donor and recipient origin (Lombardi et al 1990). Donor and recipient dendritic cells are activated in the graft and surrounding tissues and then migrate to secondary lymphoid organs. Dendritic cells interact with alloreactive naïve T cells and central memory T cells that recirculate within secondary lymphoid tissue and this circulation is controlled by chemokines and sphingosine-1-phosphate receptors (Mandala et al 2002). Naïve T cells are predominantly triggered by dendritic cells in the secondary lymphoid tissue (Lakkis et al 2000; Zhou et al 2003), but memory T cells can be activated by graft endothelium (Biederman & Pober 1998).

Full T lymphocyte activation requires 3 signals. The first signal is antigen-specific, MHC-restricted and involves triggering of the TCR by the MHC-peptide antigen complex on the antigen-presenting cell (Krensky et al 1990; Bromley et al 2001a,b), leading to clustering of the TCR/CD3 complex to CD4 or CD8 molecules and formation of the immunological synapse (Bromley et al 2001a,b). This leads to the initiation of transcription and expression of IL-2 and the IL-2 receptor.

The process of antigen recognition also results in secretion of IL-1 and IL-6 by antigen-presenting cells. The second costimulatory signal is not antigen-specific and involves both positive and negative costimulatory pathways (Janeway & Bottomly 1994; Sayegh & Turka 1998; Alegre et al 2001; Frauwirth & Thompson 2002; Greenwald et al 2002, 2005; Watts 2005). The second costimulatory signal mediates reorganization of the immunological synapse and sustains lymphocyte proliferation and differentiation into effector cells and memory cells. In the absence of the second costimulatory signal, activated T cells are unable to produce any significant amounts of cytokines (Schwartz et al 1989; Applebaum & Boussiotis 2003; Schwartz 2003), do not proliferate and become unable to respond to appropriate stimulation, i.e. anergic (Gimmi et al 1993), or undergo apoptosis (Noel et al 1996). The initial costimulatory signal involves the CD28/B7 pathway with interaction of B7-1/CD80 and B7-2/CD86 on the dendritic cells with CD28 on T cells (Bromley et al 2001b; Sharpe & Freeman 2002; Greenwald et al 2005).

The combination of first and second signal leads to activation of three signal transduction pathways (Nel 2002): i) the calcium-calcineurin pathway, ii) Ras-MAP-kinase pathway and iii) the protein-kinase C-nuclear factor-κB (NF-κB) pathway. These three pathways respectively lead to activation of the transcription factors NFAT (nuclear factor of activated T cells), activating protein 1 (AP-1) and NF-κB. This leads to expression of IL-2, IL-2 receptor, CD40 ligand and other cytokines including IL-15. IL-2 and IL-15 provide signal 3 activating mTOR (mammalian target of rapamycin) through the phosphoinositide-3-kinase pathway and initiating cell cycle progression. The cytokine γ chain which binds to Janus kinase 3 (JAK 3) initiating signal 3 is shared by a number of cytokines (IL-2, IL-4, IL-7, IL-15, IL-21) (Alves et al 2007). Lymphocyte replication and proliferation also requires purine and pyrimidine synthesis. These three signals lead to generation and proliferation of effector T cells.

The CD40/CD40 ligand pathway is another important early costimulatory pathway involved in T cell activation (Hancock et al 1996; Peng et al 1996; Van Gool et al 1996; Grewal & Flavell 1996). CD40, a member of the TNFR family, is expressed on antigen-presenting cells (Foy et al 1996; Grewal & Flavell 1998) and endothelial cells (Hollenbaugh et al 1995; Karmann et al 1995; Yellin et al 1995; Larsen & Pearson 1997; Reul et al 1997) and is stimulated by CD40 ligand (CD154) expressed on T cells including activated CD4 T cells and a subset of CD8 T cells and NK cells (Noelle et al 1992; Roy et al 1993, 1995; Larsen et al 1996; Larsen & Pearson 1997; Niimi et al 1998).

Signal 1 rapidly induces CD40 ligand expression by CD4 T cells and to lesser extent on CD8 T cells (Larsen & Pearson 1997). CD40/CD40 ligand pathway augments the CD28/B7 costimulatory pathway by upregulating B7-1/B7-2 expression on antigen-presenting cells, but is also able to independently stimulate T cells (Roy et al 1995; Van Essen et al 1995; Blotta et al 1996; Larsen & Pearson 1997). CD40 ligand signaling in the presence of IL-12 increases IFN-γ production by activated T cells (Konieczny et al 1998; McDyer et al 1998; Peng et al 1998). CD28 and CD40 ligand costimulation enhance IL-4 production (Blotta et al 1996). CD40 ligand stimulation of CD40 triggers signals for B cell antibody production and upregulates MHC and B7-1/B7-2 expression on antigen-presenting cells (Ranheim & Kipps 1993; Hancock et al 1996; Van Kooten et al 1997). CD40 ligand ligation of endothelial CD40 induces expression of adhesion

molecules, cytokines, chemokines and growth factors (Hollenbaugh et al 1995; Karmann et al 1995; Henn et al 1998; Slupsky et al 1998).

T cells also express the inhibitory CTLA-4 which like CD28 binds to B7-1 and B7-2, but with a higher affinity (Peach et al 1994; Greene et al 1996; van der Merwe et al 1997; McAdam et al 1998; Stamper et al 2001; Collins et al 2002). CD28 is constitutively expressed, but CTLA-4 expression is upregulated and is maximal 48-72 hours after TCR stimulation and inhibits TCR and CD28-mediated signal transduction (Lee et al 1998). CTLA-4/B7 pathway may also play a regulatory role involving dendritic cells (Grohman et al 2002; Finger & Bluestone 2002; Munn et al 2004). CTLA on Tregs binds to B7 molecules on dendritic cells stimulating production of IFN-γ, which acts in an autocrine and paracrine manner to stimulate indole amine 2,3-dioxygenase (IDO). IDO degrades tryptophan and this depletion together with the tryptophan degradation products inhibits T cell proliferation and promotes apoptosis (King & Thomas 2007).

CD40/CD40 ligand binding also plays in important role in dendritic cell function and macrophage activation. CD40 ligation induces antigen-presenting cells to release pro-inflammatory cytokines – IL-1β, IL-6, IL-8 and TNF-α (Kiener et al 1995), nitric oxide (Tian et al 1995) and matrix metalloproteinases (Malik et al 1998) that break down the extracellular matrix and facilitate infiltration by inflammatory cells. CD40 ligation increases antigen-presenting cell expression of ICAM-1 and CD44H and IL-12 production (Cella et al 1996; Guo et al 1996; Kato et al 1996; Koch et al 1996). CD40 ligation inhibits both spontaneous and Fas-mediated apoptosis of antigen-presenting cells (Larsen & Pearson et al 1997).

In addition to the classical positive costimulatory pathways of CD28/B7 and CD40/CD40 ligand and the negative CTLA-4 pathway (Alegre et al 2001; Saloman & Bluestone 2001), other novel costimulatory molecules playing a role include other members of the CD28/CTLA/B7 and TNFR/TNF family (Coyle & Gutiemez-Ramos 2001; Locksley et al 2001; Sharpe & Freeman 2002; Yamada et al 2002; Carreno & Collins 2003; Croft 2003; Salama & Sayegh 2003; Watts 2005). These novel costimulatory molecules differ in their timing and distribution of their expression and in the delivery of positive or negative signals. The outcome of the T cell response depends on the integration of both positive and negative signals. These signaling interactions are

not limited to T cell-antigen-presenting cell interactions, but also include T cell – T cell and T cell – B cell interactions as well as T cell signaling with non-lymphoid cells such as parenchymal cells and the endothelium (Kroczek et al 2004).

These costimulatory pathways have been targeted as potential therapeutic strategies to induce long-term allograft acceptance (Wekerle et al 2002; Snanoudj et al 2006; Weaver & Charafeddine 2008).

3. ROLE OF T CELL SUBSETS IN ALLOGRAFT REJECTION

3.1. CD4 T cell subsets

CD4 T cells are thought to be critical in the initiation of graft rejection (Krieger et al 1996). Activated CD4 T cells in both the direct and indirect pathways proliferate and secrete a variety of cytokines that are necessary to initiate the immune response (Krensky et al 1990; Lakkis et al 1998; Keane-Myers et al 1998; O'Garra 1998). The cytokines act in an autocrine manner on CD4 T cells themselves or in a paracrine manner as growth and activation factors for CD8 cytotoxic T cells, B cells and macrophages resulting in destruction of the transplanted organ by direct lysis of target cells, antibody production and delayed-type hypersensitivity mechanisms respectively (Sayegh & Turka 1998; Rocha et al 2003).

3.1.1. CD4 Th0, Th1 and Th2 cells

CD4 Th cells secrete cytokines which provide help for activation, proliferation and differentiation of other lymphocytes (Strom et al 1996; Lakkis 1998; Keane-Myers et al 1998; O'Garra 1998). At the time of activation, ***CD4 Th0*** cells secrete IL-2, IFN-γ and IL-4. Activation results in development of two major phenotypes, Th1 and Th2 CD4 cells, which differ phenotypically by the secretion of distinct cytokine profiles. ***CD4 Th1*** cells are induced by IL-12 and IFN-γ to secrete IL-2, IL-3, IFN-γ and TNF-α/β. CD4 Th1 cytokines promote immunopathologic functions by mediating delayed-type hypersensitivity (DTH), promoting generation of the cytotoxic T cells (CTLs), activating natural killer cells, activating macrophages with consequent production of nitric oxide, reactive oxygen intermediates, degradative enzymes and TNF-α. IFN-γ and

TNF-α upregulate the expression of B7 costimulatory molecules, thereby, enhancing T cell activation (Pechhold et al 1997). Th1 cells have a restricted effect on B cells, but the induction of IgG 2a antibodies with complement-fixing and opsonizing activities may contribute to rejection (Demetris et al 1992). CD4 Th1 cells also express fucosyltransferases necessary for the synthesis of glycosylated ligands for endothelial E-selectin and P-selectin that are involved in lymphocyte migration into sites of inflammation (van Wely et al 1998). ***CD4 Th2*** cells are induced by IL-4 to secrete IL-4, IL-5, IL-6, IL-10 and IL-13. CD4 Th2 cytokines provide B cell help by stimulating secretion of IgG1 and IgE antibodies. IL-10 downregulates the expression of B7 costimulatory molecules diminishing activation of T cells (Li et al 1996). Th2 cytokines (IL-5) are also able to activate and recruit eosinophils and mast cells into sites of inflammation. In the presence of IL-4 and inhibition of CD4 Th1 cell proliferation, CD4 Th2 cells can mediate IL-5 dependent eosinophilic rejection (Surquin et al 2005; Wu et al 2006).

CD4 Th1 IFN-γ inhibits Th2 cell proliferation and cytokine secretion. CD4 Th2 IL-10 inhibits Th1 proliferation and cytokine expression. There is, thus, a reciprocal regulation resulting in a dynamic balance between Th1 and Th2 profiles within sites of inflammation (Romagnani 1991; Mosmann & Sad 1996). Many factors affect the differentiation and progression of CD4 Th0 cells to either a Th1 or Th2 phenotype (Abbas et al 1996; Murphy & Reiner 2002). These factors include pro- and anti-inflammatory cytokines produced by cells of innate and adaptive immunity at the site of tissue injury, antigen concentration and binding affinities, costimulatory molecules, routes of antigen introduction and adhesion molecule upregulation. The balance between the transcription factors T-bet (IL-12) and GATA3 (IL-4) is the crucial determinant between Th1 and Th2 development respectively.

Thus, CD4 Th1 cells contribute to rejection by:

i) the provision of signals that promote the generation of CD8 CTL activity either directly by secreting IL-2 or indirectly by activating dendritic cells to promote CTL differentiation;

ii) providing help signals that promote differentiation and activation of allo-antibody producing B cells

iii) activating antigen-independent effector leukocytes which damage engrafted tissue (**delayed-type hypersensitivity, DTH**). Activated Th1 cells are critical for DTH. Through their secretion of IFN-γ and TNF-α, Th1 cells recruit and trigger macrophages to produce toxic molecules such as nitric oxide (NO), reactive oxygen intermediates, degradative enzymes and TNF-α. NO is cytotoxic at high concentrations and also causes vasodilatation, increased vascular permeability, extravasation and tissue oedema that is seen in DTH. TNF-α binds to TNFR and can induce T cell apoptosis or necrosis through caspase activation. Activated neutrophils release myeloperoxidase which in turn generates toxic metabolites such as reactive oxygen species and H_2O_2. This leads to tissue swelling and induration and the presence of an inflammatory infiltrate rich in T cells, macrophages and neutrophils characteristic of the DTH reaction. This reaction requires primed antigen-specific memory Th1 cells and alone is capable of mediating allograft rejection (Dalloul et al 1996; Valujskikh et al 1998). The immunoregulatory cytokines,TGF-β and IL-10 downregulate the DTH response and have been shown to be associated with allograft acceptance (VanBuskirk et al 2000).

Th1 cells are thought to be more responsible for allograft rejection via DTH and CTL responses. CD4 Th2 cytokines may be able to counteract acute rejection by suppressing DTH, inhibiting IFN-γ- mediated activation of macrophages and stimulating the production of non-complement fixing and non-opsonizing antibodies.

However, studies have confirmed that Th2 cytokines are not essential for prolonged graft survival and an immune response driven by both Th1 or Th2 cells is damaging to the graft, but a Th2 response may be less damaging (Strom et al 1996; Saleem et al 1996; Wasowska et al 1996; Nickerson et al 1994; 1997; Lakkis et al 1997; Rocha et al 2003; Alegre et al 2007). The difference in expression of most chemokines and costimulatory receptors between Th1 and Th2 cells is quantitative.

3.1.2. CD4 Th17 cells

CD4 Th0 cells under the influence of TGFβ, IL-6 and IL-23 develop into IL-17-secreting CD4 Th17 cells (Bettelli et al 2007; Goriely & Goldman 2007) and induce neutrophil inflammation (Weaver et al 2007). IL-27 inhibits the activity of Th17 cells (Goriely & Goldman 2007). TGF-secreting Tregs may influence Th17 cell activity

(Lohr et al 2006; Korn et al 2007; Wahl 2007). Neutrophils play an important role in acute allograft rejection especially in the setting of costimulatory blockade and in the absence of IFN-γ and IL-4 (Miura et al 2003; El-Sawy et al 2005; Surquin et al 2005).

3.1.3. Regulatory CD4 cells

A regulatory CD4 T cell phenotype has been identified and includes $CD4^+$ $CD25^+$ Tregs, IL-10-producing type I regulatory cells and murine TGF-producing Th3 cells (Groux et al 1996; Letterio & Roberts 1997; Seder et al 1998; Jiang et al 2006a; Yong et al 2007). Regulatory T cells are predominantly $CD4^+$, but other T cell subsets such as $CD8^+$ T reg (Zhou et al 2001; Gilliet & Liu 2002), veto $CD8^+$ cells, γδ T cells, NK 1.1^+ $CD4^-$ $CD8^-$ cells, NK 1.1^- $CD4^-$ $CD8^-$ cells and TCR^+ $CD4^-$ $CD8^-$ T cells have also been shown to have regulatory capacity (Ciubotariu et al 1998b; Zhang et al 2000, Seino et al 2001; Karim et al 2002; Mills 2004; Yi et al 2006).

$CD4^+$ T reg cells can be divided into two groups, the natural and the adaptive Tregs, depending on their origin (Mills 2004).

Natural $CD4^+$ $CD25^+$ T cells develop in the thymus and account for 5-10% of mature $CD4^+$ $CD8^-$ thymocytes and 10% of peripheral $CD4^+$ T cells (Itoh et al 1999; Jordan et al 2001) and are predominantly found in lymphoid organs (Cottrez & Groux 2004). There is no definitive cell surface marker as CD45 RB, CTLA-4, glucocorticoid-induced TNF-receptor family related receptor (GITR), CD122, CD103, CD134 and CD62L are all expressed on other CD4 T cell subsets especially activated T cells (Wood & Sakaguchi 2003; Yi et al 2006). FOXP3 (forkhead/winged helix transcription factor) is a key regulator for the development of Tregs and is restricted predominantly to $CD4^+$ $CD25^+$ TCR αβ cells (Fontenot et al 2003; Hori et al 2003).

In vitro studies have shown that $CD4^+$ $CD25^+$ Tregs suppress effector T cell proliferation through a cell contact dependent and largely cytokine independent mechanism (Takahashi et al 1998; Suri-Payer & Cantor 2001; Piccirillo & Shevach 2001). CTLA-4 and GITR expressed on the surface of Tregs play a role in mediating suppression of T effector function (Takahashi et al 2000; Shimizu et al 2002). However, IL-10 and TGF-β play an important role in natural Treg mediated unresponsiveness to alloantigens in *in vivo* models (Josien et al 1998; Hara et al 2001). Natural Tregs are

important in maintaining overall immune homeostasis and preventing autoreactivity (Walsh et al 2004).

$CD4^+$ $CD25^+$ regulatory cells are responsive to chemokines that engage with CCR4 and CCR8 receptors, thereby enabling regulatory cells to localize at sites of inflammation (Iellem et al 2001). $CD4^+$ $CD25^+$ regulatory cells do not proliferate in response to stimulation (Jonuleit et al 2001; Dieckmann et al 2001). $CD4^+$ $CD25^+$ regulatory cells regulate the proliferation of both $CD4^+$ (Levings et al 2001; Sanchez-Fueyo et al 2002) and $CD8^+$ cells (Piccirillo & Shevach 2001; Yamagiwa et al 2001; Sanchez-Fueyo et al 2002). The suppressive effect is dependent on TCR stimulation of the regulatory cells and direct cell contact with the target cells and is not antigen-specific (Thornton & Shevach 2000; Yamagiwa et al 2001). $CD4^+$ $CD25^+$ regulatory cells are able to suppress polyclonally- and alloantigen-stimulated T cell proliferation (Levings et al 2001; Stephens et al 2001; Taams et al 2001). IL-2 is an important growth factor for $CD4^+$ $CD25^+$ regulatory cells (Jonuleit et al 2001; Dieckmann et al 2001).

$CD4^+$ $CD25^+$ regulatory cells constitutively express CTLA4 in both mice (Takahashi et al 2000; Read et al 2000; Salomon et al 2000) and humans (Jonuleit et al 2001; Dieckmann et al 2001; Stephens et al 2001; Levings et al 2001).

There are two populations of adaptive Tregs that are important in the development of transplantation tolerance (Walsh et al 2004; Kang et al 2007): the Th3 cells (mice) that secrete TGFβ in response to ingestion of antigen in the development of oral tolerance (Chen et al 1994) and Tr1 cells (human) that secrete large amounts of IL-10 (Groux et al 1997).

Adaptive Tregs depend on peripheral factors for their development such as the availability of TGFβ and the maturity and type of stimulating antigen-presenting cell (Steinman et al 2003). Adaptive Tregs do not depend on cell-contact, but suppress immune responses through immunosuppressive cytokines such as IL-10 and TGF-β (Stassen et al 2004). Tr1 cells migrate to sites of inflammation (Cottrez & Groux 2004). Adaptive Tregs are identified according to secretion of cytokines. Dendritic cells are able to induce a Th3 or Tr1 Treg phenotype irrespective of their maturation state (Wakkach et al 2003; Lavelle et al 2003). In addition, Tregs have been shown to modulate dendritic cells, downregulating the expression of MHC class II, costimulatory

molecules and IL-12 (Kang et al 2007). Tregs prevent interactions between dendritic cells and T effector cells that are essential for effective priming of T effector cells (Tadokoro et al 2006).

$CD4^+$ $CD25^+$ Tregs play an important role in inducing effector T cells to adopt a Tr1 suppressor phenotype (Jonuleit et al 2002; Dieckmann et al 2002). $CD4^+$ $CD25^-$ $FOXP3^-$ effector cells through stimulation via TCR in the presence of IL-10 and TGFβ can develop into $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs (Fu et al 2004; Bushell et al 2005).

Adaptive Tregs are thought to develop and act directly within the graft (Josien et al 1998). The predominant mode of allorecognition by Tregs is via the indirect pathway (Caballero et al 2006). Both natural Tregs and adaptive Tregs are probably necessary for the induction of transplantation tolerance (Cottrez & Groux 2004; Waldmann et al 2004; Walsh et al 2004). Clinical transplantation tolerance procedures need to delete alloreactive T cells and spare/amplify Tregs (Lechler et al 2003).

3.2. Cytotoxic T lymphocytes

CTLs are primarily derived from CD8 T cells, but CD4 T cells may also be cytotoxic after they are activated by the indirect allorecognition pathway (Shresta et al 1998). CD8 CTLs are primed and activated by direct presentation of donor MHC class I antigens by donor APCs (Arakelov & Lakkis 2000; Barry & Bleackley 2002; Russel & Ley 2002) or by activated donor vascular endothelial cells (Kreisel et al 2002). The requirement for costimulatory molecules is controversial as different studies using CD40-CD40 ligand blockade have shown conflicting results for CD8 CTL activation (Jones et al 2000; Zhai et al 2003). Activated CD8 T cells primarily damage grafts by direct cytolysis of graft parenchymal or vascular cells bearing antigens which the CTL's TCR recognizes. CD8 T cell cytolysis is initiated by direct presentation of foreign MHC-I molecules. The mature cytoxic CD8 T cells secrete IFN-γ and TNF-α. Two cytolytic mechanisms for CTLs have been identified. These include secretory (granule exocytosis) and non-secretory (Fas/Fas-ligand) pathways which are calcium-dependent and calcium-independent, respectively (Liu et al 1996; Kagi et al 1996; Russel & Ley 2002; Barry & Bleackley 2002). In the secretory mechanism, antigen-specific CTLs synthesise cytolytic granules containing granzymes, perforin, granulysin, serglycin,

calreticulin and Fas ligand within 24 – 48 hours following activation. Antigen-specific CTLs tightly engage their target cells and release the cytolytic granules that flow vectorially to the site of contact and fuse with the target cell membranes (Yannelli et al 1986). In the presence of calcium, perforin polymerises and forms pores within the target cell membrane, facilitating access of cytolytic proteins to the cytoplasm and promoting osmotic lysis. Granzymes trigger apoptosis of the target cell by activating a series of cysteine-containing aspartate-specific proteases (caspases) either by direct cleavage of procaspase-3 or by indirect activation of procaspase-9 and subsequent cleavage of procaspase-3 (Chinnaiyan et al 1996a; Sutton et al 2000; Wang et al 2001). Granulysin induces target cell lysis by the generation of intracellular ceramide from sphingomylein or as a result of activation of caspases (Pena & Krensky 1997). In the non-secretory, calcium-independent mechanism, Fas ligand (FasL) is upregulated on the cell surface membrane of CTLs within several hours of antigen-specific activation. The FasL-bearing CTL is able to engage Fas (CD95) expressed constitutively or induced by cytokines on the cell surface of target cells. The binding of FasL to the Fas receptor leads to the aggregation of intracellular death domains and the recruitment of Fas-associated death domains and pro-caspase-8 to form a death-inducing signaling complex that results in apoptosis (Boldin et al 1995; Chinnaiyan et al 1995, 1996b; Kischkel et al 1995). CD8 CTLs use both the perforin/granzyme and FasL pathways of cytotoxicity, but CD4 CTLs preferentially use the FasL pathway (Shresta et al 1998). CD8 T cells may produce signals that have cytotoxic action or mediate DTH through the secretion of IFN-γ in the vicinity of target cells. Effective stimulation of CD8 cytotoxicity is thought to require costimulation by CD40:CD40L in a CD4 dependent fashion, but there have been conflicting studies (Jones et al 2000; Zhai et al 2003). Other alternative costimulatory molecules, such as LFA-1, LIGHT and membrane lymphotoxin may influence CTL activation (Guo et al 2001; Scheu et al 2002; Corbascio et al 2002).

CD8 T cells express chemokine receptors including CXCR3 and CX3CR1 and secrete a large number of chemokines such as CXCL8 (IL-8), CXCL10 (IP-10), CCL3 (MIP-1α), CCL3, CCL4 (MIP-1β) and CCL5 (RANTES), thereby establishing a chemokine gradient to recruit other effector cells to the site of injury (El-Sawy et al 2002).

3.3. NK cells

NK cells are large granular leukocytes which are able to kill virus-infected or tumour cells without prior antigen-specific activation (See et al 1997; Lanier 1998a; Biron et al 1999; Cerwenka & Lanier 2001). NK cells have a unique mechanism of lysis as they do not express TCRs or any molecules that are able to recognise antigen. They do not require prior sensitization and are not antigen-specific.

NK cells express both inhibitory self MHC-I specific killer Ig-like receptors (KIRs) and triggering natural cytotoxicity receptors (Timonen 1997; Lanier 1998a,b,c; Ryan et al 2001; Middleton et al 2002; McQueen & Parham 2002; Moretta et al 2002). NK cell activation is controlled by the balance of signals received from the engagement of these 2 receptors.

NK cells kill those target cells that lack or express insufficient self MHC class I molecules by cell-to-cell cytotoxicity (Liu et al 1996; Kagi et al 1996; Russel & Ley 2002; Barry & Bleackley 2002). NK cells lyse target cells via the granzyme exocytosis pathway (Shresta et al 1995). NK cells also express a low affinity receptor for the Fc portion of IgG (CD16) and bind to and kill antibody-coated target cells (antibody-dependent cell cytotoxicity) as well as secreting TNF-α which may induce apoptosis of TNFR-expressing graft cells. Activated NK cells express chemokine receptors CXCR1 and CXCR3 and release numerous cytokines and chemokines leading to mononuclear cell recruitment and activation.

NK cells are important effector cells mediating rejection of allogeneic haemopoietic cells (Murphy et al 1987; Manilay & Sykes 1998), and their role in mediating both solid organ graft rejection and tolerance as well as their role in xenograft rejection is now recognized (Kitchens et al 2006).

NK cells appear to play a significant role in allograft rejection in the setting of co-stimulatory blockade (Maier et al 2001). NK cells may facilitate acute rejection via a number of mechanisms including:

i) NK cells are able to promote a CD4 Th1 phenotype directly, enhancing antigen-specific T cell proliferation (McNerney et al 2006) and IFN-γ production or indirectly via enhancement of dendritic cell maturation (Gerosa et al 2005; Moretta et al 2006).

ii) Activated NK cells secreting IFN-γ can upregulate MHC molecule expression on endothelial cells, thereby enhancing endothelial cell recognition by alloreactive T cells (Gerosa et al 2005; Moretta et al 2006).

iii) NK cells infiltrating allografts secrete the early chemokines (MIP-1α, MIP-1β, CXC1, CCL3, CXCL10 and CX3CL1) that attract the alloreactive T cells (Kondo et al 2000; Obara et al 2005).

NK cells lacking the inhibitory Ly49 receptors for donor MHC class I molecules promote rejection, whereas Ly49G2+ NK cells suppress alloimmune responses (McNerney et al 2006).

NK cells have been shown to be clinically relevant in acute rejection in renal transplantation (Fuggle 1991; Blancho et al 1992; Kummer et al 1995) and liver transplantation (Bishara et al 2001). NK cells play an important role in xenograft rejection (Seebach & Waneck 1997; Rieben & Seebach 2005). Failure of NK inhibitory receptors to recognize xenogeneic MHC class I molecules leads to NK cell activation with either direct perforin mediated damage or cytokine-induced xenoreactive T cell activation (Schneider et al 2001; Xu et al 2002). NK cells promote a pro-inflammatory endothelium, thereby recruiting inflammatory cells (Goodman et al 1996; Dawson et al 2000). ADCC also occurs in response to xenoreactive antibodies binding to galactose-α (1,3)- galactose on the xenograft (Sandrin et al 1993).

NK cells may also be able to promote induction of allograft tolerance in the setting of costimulatory blockade. This may involve perforin dependent downregulation of CD4 and CD8 cells in the alloimmune response (Beilke et al 2005) or the destruction of donor antigen-presenting cells (Yu et al 2006).

3.4. Memory T cells

Alloreactive memory T cells differ from naïve T cells in gene expression profile, costimulatory requirements for activation, homeostasis and homing properties, and are more resistant to tolerance induction (Chalasani et al 2002; Sprent & Surh 2002). Effector memory T cells require minimal costimulation signaling from B7 and CD40 on APCs (London et al 2000). Targetting of alternative co-stimulatory receptors such as the CD27/CD70 pathway has resulted in longterm cardiac allograft survival in CD28-deficient mice (Yamada et al 2005). Memory T cells have been shown to be a major barrier to the successful induction of tolerance (Brook et al 2006; Valujskikh 2006). This is irrespective of the mechanism of generation of memory cells including: i) heterologous immunity where memory cells are generated following prior exposure to allo-antigens or to micro-organisms that induce cross-reactive memory T cells (Adams et al 2003), ii) homeostatic proliferation of memory T cells following partial T cell depletion (Wu et al 2004). Thus, present immunosuppressive protocols that result in partial T cell depletion may promote memory T cell generation and prevent tolerance induction. Experimentally this has been overcome by the transfer of unprimed regulatory cells following T cell depletion therapy or by the addition of non-depleting anti-CD4 and anti-CD8 antibodies that reduce memory cell homeostatic proliferation (Neujahr et al 2006). Outbred large animals living in non-pathogen-free environments and humans have a large number of memory type alloreactive T cells that are cross-reactive to previously sensitised environmental antigens. Thus, treatment protocols capable of inducing longterm tolerance in rodents, e.g. combination of anti-CD154 and donor-specific transfusion may fail in large animal models or primed recipients (Zhai et al 2002). Depletion of memory CD8 T cells in combination with anti-CD154 antibody and global immunosuppression has been shown to induce mixed chimerism and tolerance in a non-human primate model of donor bone marrow/kidney transplantation (Koyama et al 2007). The precursor frequency of CD8 T cells determines their requirement for CD28/CD154 costimulation. High antigen-specific precursor frequency CD8 T cells are less dependent on the CD28/CD154 costimulation pathways for full activation (Ford et al 2007). Thus, clinical transplant tolerance may need therapeutic regimens that tolerise distinct memory CD4 and CD8 T cell subsets (Kaech & Ahmed 2001; Zhai et al 2002).

4. ALLOANTIBODY RESPONSES

B cells bind soluble antigens via surface immunoglobulins, indirectly process them into peptides and transport them into the groove of surface MHC II molecules. These CXCR5-expressing antigen-primed B cells along with a subset of activated CD4 Th cells migrate to B cell follicles in response to the B cell ligand CXCL13 expressed on follicular stromal cells (Mackay 2001; Moser & Loetscher 2001). During T cell – B cell interaction, the indirectly primed CD4 Th cells recognize MHC II-peptide complexes expressed on B cells and provide costimulatory signals through CD40L which binds to CD40 on the B cell. These Th cell costimulatory signals play a central role in B cell activation, proliferation and differentiation into terminally differentiated antibody-producing plasma cells and memory B cells, allo-antibody production and isotype and subclass switching (Foy et al 1996; Lederman et al 1996; Lipsky et al 1997; Fink & McMahan 2000; Calame et al 2003). Allo-antibodies circulate and reach the allograft through the endothelial pores. Graft cells coated with antibodies can be killed by activation of the complement cascade or by perforin/granzyme-mediated NK cytotoxicity (i.e. antibody-determined cell mediated cytotoxicity (ADCC)) (Baldwin et al 1995, 2001). The severity of tissue injury caused by these antibodies depends on the isotype, affinity and antibody titre as well as the expression of target antigen. It has been shown that IgG1 allo-monoclonal antibodies to MHC class I antigens can augment graft injury by stimulating endothelial cells to produce MCP-1 and by activating mononuclear cells through their Fc receptors (Lee et al 2007). Graft-infiltrating cells such as macrophages, neutrophils and eosinophils are also activated by the crosslinking of their FcRs by allo-antibodies and complement.

Anti-donor antibodies present at the time of transplant are able to trigger hyperacute rejection. Preformed antibodies directed against ABO-blood group carbohydrate antigens, donor MHC class I and to a lesser degree MHC class II, as a result of previous blood transfusions, pregnancy or previous transplantation, immediately bind to the vascular endothelium of the graft after revascularization. These preformed antibodies rapidly activate the complement system and the coagulation cascades leading to intravascular thrombosis, haemorrhagic necrosis and graft infarction.

Graft infiltrating B cells are also able to present graft-derived peptides, thereby locally activating alloreactive T cells via the indirect pathway of allorecognition (Alegre et al 2007).

5. BLOCKADE OF CO-STIMULATING PATHWAYS AND TOLERANCE INDUCTION

T cell costimulation is mediated by a network of signaling pathways. The various co-stimulatory pathways play different roles: some provide the classical costimulatory signals required to initiate priming of naïve T cells, others are necessary to sustain T cell activation and others provide signals to downregulate T cell responses. Blockade of these various pathways can result in many different tolerance mechanisms including immune regulation, anergy and deletion (Wekerle et al 2002; Snanoudj et al 2006; Weaver et al 2008). However, immune tolerance, a state of unresponsiveness to the donor graft (Sykes et al 2007) is difficult to achieve (Salama et al 2007). Disruption of a single pathway is usually not sufficient to promote tolerance or long-term allograft survival. Clinical success is more likely to be obtained by the blockade of a number of co-stimulatory pathways.

5.1. Blockade of CD28/CTLA-4 : B7 pathway

Most experimental models have looked at the role of CTLA4-Ig or monoclonal antibodies blocking CD28 interaction with its ligands B7-1 (CD80) and B7-2 (CD86), as well as anti-CD154 mAbs to block CD40:CD40L (CD154) interactions. These models rely on the eventual deletion of alloreactive T cells, as in the absence of costimulation which induces cell survival genes, T cells become susceptible to the induction of apoptosis (Dai et al 1998; Wells et al 1999). CD4 cells are more susceptible to costimulatory blockade than CD8 cells, and in some models, costimulatory blockade induces tolerance only in conjunction with CD8 depleting agents (Trambley et al 1999; Jones et al 2000).

CTLA4 (CD152) plays a role in inducing clonal anergy, reducing the number and activity of CD4 Th1, Th2 and CD8 T cells responding to an allogeneic challenge (Frauwith et al 2000, 2001; Greenwald et al 2001; Wells et al 2001; Clarkson & Sayegh

2005). CTLA4 is needed for the induction of a division-arrest form of anergy (Wells et al 2001), but is not required for anergy through CD28-blockade (Frauwith et al 2000; Wells et al 2001) and is not essential for anergising CD8 cells (Frauwith et al 2001). CTLA plays an important role in tolerising CD4 cells *in vivo* (Greenwald et al 2001).

A recombinant fusion protein, CTLA4-Ig, which consists of the extracellular binding domain of CTLA4 linked to a modified Fc domain of human IgG1, binds with a higher affinity than CD28 to B7 molecules and thereby acts as a potent competitive antagonist of the CD28:B7-mediated T cell costimulation (Linsley et al 1991). The administration of CTLA4-Ig resulted in longterm survival of xenogeneic human-to-mice pancreatic islet grafts (Lenschow et al 1992) as well as prolonged survival of rat cardiac allografts (Turka et al 1992). However, a true state of tolerance was not obtained, as all the grafts were ultimately rejected. Subsequent studies in MHC mismatched cardiac allografts showed that the administration of donor specific transfusion (donor splenocytes) on the day of transplantation, followed by a single injection of CTLA4-Ig on day 2 post transplantation, was effective in achieving tolerance (Lin et al 1993). It has subsequently been confirmed that donor antigen in conjunction with CTLA4-Ig or prolonged blockade is required in cardiac allograft models for the induction of tolerance and prevention of chronic allograft vasculopathy (Pearson et al 1996; Sayegh et al 1997). The role of donor cells in the promotion of tolerance induction in these animal models is not known.

However, the timing of the administration of CTLA4-Ig is important and has been shown to be most effective, if CTLA4-Ig is administered after transplantation (Lin et al 1993; Sayegh et al 1995). It is thought that this may be due to the fact that cycling T cells are more susceptible to the absence of co-stimulatory signals than resting T cells (Sayegh & Turka 1998). In addition, T cells express CTLA4 on their surface after activation and this transduces a negative regulatory signal to the cell (Walunas et al 1996). Thus, delaying the administration of CTLA4-Ig would enable B7 molecules to bind to CTLA4, which may be required for the induction of tolerance (Perez et al 1997).

Several studies of transplantation in rats, mice and non-human primates including studies of cardiac, renal, liver, intestine and islet allografts, have confirmed that blockade of the CD28:B7 pathway has resulted in prolonged graft survival and in some

instances donor-specific tolerance (Lenschow et al 1992; Turka et al 1992; Lin et al 1993; Sayegh et al 1995; Levisetti et al 1997; Schaub et al 1998; Newell et al 1999).

Both CTLA4-Ig and anti-CD154 are most effective when used together with donor-specific transfusion (Lin et al 1993; Markees et al 1997). Donor-specific transfusion and anti-CD154 results in substantial prolongation of MHC-mismatched skin grafts, but permanent graft survival is only achieved in a small percentage of euthymic recipients (Markees et al 1997). However, with the same treatment, permanent graft survival can be achieved in most thymectomised mice (Markees et al 1998). In this model, tolerance induction is dependent on the continuous presence of CD4 cells and could be blocked by anti-CD152 and anti-IFNγ mAbs administered at the time of tolerance induction (Markees et al 1998). Clonal deletion of alloreactive T cells plays a prominent role in this regimen and CD8 depletion can substitute for donor-specific transfusion in this model (Iwakoshi et al 2000).

Recently, the use of a monoclonal antibody that modulates CD28 prevented acute and chronic renal allograft rejection in a murine model. This was associated with the generation of TCR class II B7 regulatory cells (Haspot et al 2005).

i) The role of B7-1 and B7-2 in allograft rejection

Blockade of B7-1 (CD80) or B7-2 (CD86) in acute rejection models has varying outcomes (Lenschow et al 1995; Hancock et al 1996; Zheng et al 1997). Anti-B7-2 mAb prolongs allograft survival in mice, especially when administered at time of transplantation, but anti-B7-1 has little effect (Lenschow et al 1995; Judge et al 1999). Anti-B7-1 mAb has been shown to accelerate cardiac allograft rejection in $CD28^{-/-}$ mice by blocking CTLA4 interaction (Yamada et al 2001). In contrast, anti-B7-2 mAb, in the absence of CD28, prolongs engraftment (Yamada et al 2001).

The B7/CTLA-4 interaction is necessary for the CD4+ CD25+ Treg function (von Boehmer 2005) and in a skin transplant model, CTLA-4 pathway blockade prevented the regulatory activity of CD4+ CD25+ T cells (Kingsley et al 2002).

In non-human primate studies, targeting B7-1 and B7-2 either with blocking mAbs or with CTLA4-Ig prolongs both renal and islet cell allograft survival. However, the immunosuppressive effect requires the simultaneous blockade of CD40:CD154

signaling or the concomitant administration of rapamycin (Kirk et al 1997, 2001b; Adams et al 2002; Birsan et al 2003). LEA29Y (Belatacept), a high affinity variant of CTLA4-Ig has shown promise as an immunosuppressive agent in non-human primate studies (Adams et al 2005). It acts synergistically with anti-CD40 to prolong islet allograft survival (Adams et al 2005). A Phase II multicentre clinical trial has demonstrated efficacy in preventing acute rejection and chronic allograft nephropathy in renal transplant patients (Vincenti et al 2005) and Phase III clinical trials have begun.

In non-human primates, costimulatory blockade with anti-CD154 mAb is more effective than CTLA4-Ig. Anti-CD154 is able to induce T cell IL-10 production and apoptosis (Blair et al 2000). Dendritic cells, macrophages and endothelial cells express CD40 and blockade of the CD40:CD154 pathway inhibits activation of these cells, blocks the expression of adhesion molecules and suppresses the secretion of inflammatory cytokines (Kirk et al 2001a; Adler & Turka 2002).

5.2. Blockade of the CD40:CD40L pathway

CD40 blockade using anti-CD154 promotes longterm allograft survival in a number of murine cardiac and pancreatic islet transplant models (Parker et al 1995; Larsen et al 1996a) and synergises with CD28 blockade (Larsen et al 1996b; Hancock et al 1996; Elwood et al 1998). Effective CD40 blockade requires prolonged administration to enable permanent engraftment of islet cell and cardiac allografts (Parker et al 1995; Hancock et al 1996). The addition of donor antigen at the time of transplantation increases the number of recipients achieving longterm engraftment, promotes donor-specific tolerance and can prevent chronic arteriopathy in cardiac transplant models (Parker et al 1995; Hancock et al 1996; Markees et al 1997). Donor specific tolerance with CD40 blockade depends on the presence of CD4 cells, IFN-γ and CTLA-signaling (Markees et al 1998).

The combination of CTLA4-Ig with anti-CD40 has been successful in promoting longterm acceptance of rat skin and cardiac allografts (Larsen et al 1996b) and prolonged renal allograft survival in a non-human primate model (Kirk et al 1997; Pearson et al 2002).

Promising results have been obtained using anti-CD154 monotherapy in non-human primate studies. Prolonged anti-CD154 therapy can result in prolonged renal and pancreatic islet allograft survival (Kirk et al 1997, 1999; Kenyon et al 1999a, 1999b). This regimen induces prolonged immunosuppression rather than true tolerance as withdrawal of therapy has eventually led to allograft rejection. In non-human primates with cardiac transplants, blockade of CD40 ligand with a humanised monoclonal antibody was effective in delaying acute rejection (Chang et al 1997).

AB1793, a novel human anti-human CD154 mAb prevented rejection of renal allografts in cynomologous monkeys (Schuler et al 2004).

Recently it has been shown that the combination of anti-CD154/anti-CD28 costimulation blockade together with rapamycin resulted in prolonged induction of chimerism following stem cell transplantation (Kean et al 2007).

Anti-CD154 mAb therapy has been complicated by the development of thrombo-embolic events in both non-human primate studies and phase I human clinical trials (Kawai et al 2000; Schuler et al 2004). In an attempt to avoid the thrombo-embolic complications associated with CD154 blockade, a combination of antagonistic antibodies against CD40 and CD86 were evaluated and found to prevent renal allograft rejection in non-human primates (Haanstra et al 2003; 2005). Recently a chimeric-anti-CD40 mAb that synergises with Belatacept (a high affinity variant of CTLA4-Ig) has been shown to prolong islet cell allograft survival in non-human primates (Adams et al 2005).

5.3. Blockade of B7-H1(PD-L1)/B7-DC(PD-L2):PD-1 pathway

B7-H1/B7-DC: PD-1 pathway plays an important role in terminating the alloimmune response either through negative signaling leading to anergy or apoptosis (Clarkson & Sayegh 2005) or through the expansion of $CD4^{+}CD25^{+}FOXP3^{+}$ Tregs (Krupnick et al 2005).

Stimulating PD-1 negative signals using a dimeric PD-L1 Ig fusion protein together with CD154 blockade induced longterm murine islet allograft survival (Gao et al 2003).

In a murine cardiac allograft model, PD-1, PD-L1 and PD-L2 are all upregulated during rejection despite the administration of cyclosporin or rapamycin. PD-L1 Ig or PD-L2 Ig alone had no effect on allograft rejection in wild-type recipients. However, the administration of PD-L1 Ig in $CD28^{-/-}$ recipients or in combination with subtherapeutic cyclosporin significantly enhanced cardiac allograft survival. The combination of PD-L2 Ig and cyclosporin had no effect (Ozkaynak et al 2002).

5.4. Blockade of ICOS:ICOS-L pathway

ICOS signaling is required for the activation and function of T effector cells (Coyle et al 2000). In the transplant setting, studies have shown that the functional consequences of ICOS signaling or inhibition are influenced by both the nature of the immune response and the timing of the therapeutic intervention with ICOS blockade.

In a murine cardiac allograft model, ICOS expression is upregulated on graft-infiltrating cells during rejection. Administration of blocking ICOS mAb or ICOS-Ig fusion protein prolonged cardiac allograft survival (Ozkaynak et al 2001; Kosuge et al 2003). Anti-ICOS antibody therapy decreased CD4 and CD8 T cell graft infiltration and prevented upregulation of ICOS mRNA (Tafuri et al 2001). Combination therapy of low dose cyclosporin and anti-ICOS antibody led to long-term murine cardiac allograft survival (> 100 days) (Ozkaynak et al 2001).

Combination therapy with ICOS Ig and CTLA4 Ig resulted in long-term acceptance of murine cardiac allografts and also donor-specific tolerance shown by acceptance of donor, but not third party skin grafts. ICOS Ig or anti-ICOS antibody alone prolonged survival time, but all cardiac allografts were eventually rejected (Kosuge et al 2003). Combination therapy of anti-ICOS antibody and subtherapeutic FK506 in a murine model of intrahepatic islet allografts prevented islet allograft rejection (Nakamura et al 2003). In a rat liver transplant model, anti-ICOS antibody prolonged graft survival and reduced ICOS expression on graft infiltrating T cells (Guo et al 2002).

Therapeutic ICOS blockade during the effector phase is more effective in promoting longterm cardiac allograft survival than early blockade at the time of transplantation in MHC mismatched recipients (Harada et al 2003; Kashizuka et al 2005). This beneficial

effect was independent of CD28 costimulation, but not of CTLA4 signaling (Harada et al 2003).

Anti-ICOS mAb synergises with rapamycin to prolong murine islet cell allograft survival (Nanji et al 2004). ICOS-B7h blockade synergises with donor specific transfusion to promote longterm survival of MHC class II mismatched skin grafts (Sandner et al 2005). This synergism is associated with a reduction in alloreactive clone size due to apoptosis of host allo-antigen specific CD4 T cells (Sandner et al 2005).

Combined blockade of CD154 and ICOS prevented chronic arterial vasculopathy and led to longterm murine cardiac allograft survival (Guillonneau et al 2005) and tolerance in a murine islet allograft model (Nanji et al 2006).

5.5. Blockade of OX-40:OX-40L (CD134:CD134L) pathway

In alloimmune responses, blockade of the OX40:OX40 ligand pathway synergises with CD28 blockade to promote longterm survival of rodent skin and cardiac allografts (Yuan et al 2003). OX40:OX40L pathway appears to play a role in mediating memory/effector responses as a combination of OX40:OX40L and CD28 blockade is able to prevent rejection in a presensitised model using adoptive transfer of primed lymphocytes into athymic heart transplant recipients (Yuan et al 2003). OX40:OX40L pathway plays an important role in CD28/CD154-independent rejection (Demirci et al 2004; Vu et al 2004).

Blockade of OX40:OX40L interaction ameliorates GVHD in a murine model of allogeneic bone marrow transplantation (Tsukada 2000).

5.6. Blockade of LIGHT:HVEM pathway

LIGHT costimulates T cell proliferation and is required for dendritic cell-mediated allogeneic T cell responses (Tamada et al 2000). In a murine cardiac allograft rejection model, combination therapy with HVEM-Ig and Cyclosporin enhanced mean allograft survival (21 days) compared to HVEM-Ig (mean 7 days) or Cyclosporin alone (10 days) (Ye et al 2002). HVEM-Ig decreased graft arterial disease in a murine cardiac allograft model (Kosuge et al 2004).

5.7. Blockade of membrane lymphotoxin (mLT)

Membrane lymphotoxin is an important regulatory molecule for CD8 T cells mediating rejection. Blockade of this regulatory molecule can be used to inhibit costimulation blockade-resistant CD8 T cell mediated allograft rejection (Guo et al 2001).

Blockade of mLT by a fusion protein LTβR-Ig or anti-mLT mAb inhibited CD8 T cell mediated rejection of murine intestinal allografts. This was associated with decreased MIG (monokine-induced by IFN-γ) and secondary lymphoid chemokine gene expression within the allografts and spleen respectively. Blocking mLT did not inhibit CD4 T cell mediated rejection (Guo et al 2001). Combination of CTLA4-Ig and LTβR-Ig inhibited CD4 and CD8 T cell mediated rejection in a murine intestinal allograft model (Guo et al 2001).

5.8. Blockade of CD27:CD70 pathway

Anti-CD70 mAb alone or in combination with anti-OX40L mAb had no effect on murine islet allograft survival (Wu et al 2001b). Anti-CD70 mAb did prolong cardiac allograft survival in wild-type recipient mice and led to indefinite cardiac allograft survival in $CD28^{-/-}$ mice (Yamada et al 2005). Blockade of CD27:CD70 signaling synergises with interruption of CD28 signaling and mediates its effects via modulation of effector/memory alloreactive CD8 T cell activation (Clarkson & Sayegh 2005; Yamada et al 2005).

5.9. Blockade of CD137 (4-1BB):CD137L (4-1BBL) pathway

The CD137:CD137L pathway is important for CD8 effector cell proliferation and survival (Shuford et al 1997; Pulle et al 2006). CD137 blockade inhibited $CD8^{+}$ but not $CD4^{+}$-mediated rejection of murine intestinal allografts (Wang et al 2003). CD137 blockade could possibly be used as adjunctive therapy for blocking CD8 T cell driven CD28-independent alloimmune responses (Salama & Sayegh 2003; Clarkson & Sayegh 2005).

6. ROLE OF ADHESION MOLECULES, CYTOKINES, CHEMOKINES AND THE ENDOTHELIUM IN ALLOGRAFT REJECTION

6.1. Lymphocyte-endothelium interactions

Adhesion molecules act as receptors and counter-receptors (ligands) in the interactions between effector cells and target cells, the migration of leukocytes and adhesion of leukocytes to endothelial cells and the extracellular matrix (Adams et al 1989, 1996; Springer 1990a,b, 1994; Butcher 1991; Albelda et al 1994; Wardlaw et al 1996; Lautenschlager et al 1996, 1997; Cavenagh et al 1998; Fuggle & Koo 1998; Choi et al 2004; Kelly et al 2007; Rao et al 2007). Adhesion molecules play a critical role in the lymphocyte/endothelial cell interactions involved in solid organ rejection (Lawrence & Springer 1991; Springer 1994; Briscoe et al 1998). With the release of inflammatory mediators such as cytokines from activated endothelium, the endothelium changes from an anti-adhesive to a pro-adhesive state (Bevilacqua 1993). The interaction between leukocytes and activated endothelial cells results in low affinity binding and rolling of leukocytes along the endothelium, with the circulating lymphocyte being tethered to activated endothelium predominantly by means of selectin-mediated interactions (Lawrence & Springer 1991; Lasky 1992). α4 integrins ($\alpha4\beta7$ and $\alpha4\beta1$/VLA-4) also play a role in the initial capture of lymphocytes (Steeber & Tedder 2000; Steeber et al 2005). Tethering enables the beta-chemokines such as RANTES, MCP-1 and MIP-1α and β to bind to G-protein coupled lymphocyte receptors, thereby triggering expression of activated integrins such as LFA-1, VLA-1 and VLA-4, which results in strong adhesion of the lymphocytes to the endothelial wall (Schall 1991; Tanaka et al 1993; Taub et al 1993a,b; Adams et al 1994). VLA-4 must form a bimolecular complex with CD44 in order to mediate firm adhesion of activated T cells (Nandi et al 2004). Important adhesion molecules on the endothelial cells belong to the Immunoglobulin supergene family and include ICAM-1, 2 and 3, platelet-endothelial cell adhesion molecule (PECAM-1) and VCAM-1. Transendothelial migration occurs under the influence of pro-migratory factors such as MIP-1β and involves interactions between lymphocyte integrins VLA-4 and LFA-1 with the basal membrane and ICAM-1 respectively and is facilitated by the digestion of ECM by leukocyte metalloproteinases.

Once lymphocytes have migrated into the interstitium, the lymphocyte is able to interact with the antigenic peptide. T lymphocyte activation follows engagement of antigen by the TCR, followed by the second important costimulatory signal. This adhesion cascade is involved in the recruitment of alloreactive T cells into allografts (Briscoe et al 1998; Fujisaki et al 1998). Studies have shown that activated endothelial cells participate in allograft rejection as a result of: i) selective recruitment of CD4 T cell subsets, ii) costimulation of T cells and resulting production of cytokines, iii) involvement in the signaling pathways of CD40:CD40 ligand, Fas:FasL and the TNF/TNFR family (Briscoe et al 1998). Antigen activation of T cells leads to differentiation into effector T cells or memory T cells. The recruitment of differentiated T cells to peripheral sites of inflammation is mediated by the interaction of adhesion molecules on activated endothelial cells of the microvasculature with ligands expressed by activated, but not naïve T cells. Memory T cells exclusively express ligands for endothelial E-selectin and P-selectin (Lichtman et al 1997). Episodes of rejection have been shown to be preceded by endothelial cell expression of E-selectin, but vascular cell adhesion molecule 1 (VCAM-1) is only expressed on endothelial cells at the time of T cell infiltration into tissues (Briscoe et al 1995). It has been shown that CD4 Th1 cells express functional selectin ligands, but CD4 Th2 cells do not, thereby explaining the selective recruitment of CD4 Th1 cells into sites of DTH reactions (Lichtman & Abbas 1997; Borges et al 1997; Tietz et al 1998; Austrup et al 1997). Chemokine expression by endothelial cells stimulated by ischaemia-reperfusion injury and cytokines also plays a role in the selective recruitment of T cell subsets in allograft rejection (Kondo et al 1996, 1997; Fairchild et al 1997; Nadeau et al 1995; Nagano et al 1997). CD4 Th1 and Th2 subsets express different types of chemokine receptors and thus endothelial expression of chemokines also contributes to selective diapedesis of T cell subsets.

Activated endothelial cells are able to costimulate the enhanced IL-2 secretory response of CD4 T cells required for proliferation and differentiation (Sperling & Bluestone 1996; Briscoe et al 1998) predominantly via endothelial LFA-3 (CD58) interacting with the T cell receptor CD2 (Hughes et al 1990; Savage et al 1991). Endothelial cell expression of B7 is able to costimulate through the CD28/CTLA4 pathways (Sperling & Bluestone 1996; Strom et al 1996; Hancock et al 1996). Endothelial cells also costimulate effector cytokine (IL-2, IL-4 and IFN-γ) production (Briscoe et al 1997; Ma & Pober 1998). Endothelial cells express CD40 (Karmann et al 1995; Hollenbaugh et al

1995; Yellin et al 1995), as well as low levels of CD40 ligands (Reul et al 1997; Mach et al 1997). Endothelial cells are able to induce CD40 ligand expression by CD4 T cells either through TCR recognition of MHC-class II-alloantigen complexes or T cell (CD2)–endothelial (CD58, LFA-3) interactions (Karmann et al 1996). CD40 ligands expressed by T cells are able to activate endothelial cell expression of adhesion molecules and cytokines (Noelle et al 1992; Hollenbaugh et al 1995). Thus, bidirectional CD40:CD40 ligand costimulation of endothelial cells and T cells may be a critical factor in allograft rejection (Vierling 1999). This is supported by studies which show that i) CD40 is primarily expressed by endothelial cells during allograft rejection (Reul et al 1997); ii) inflammatory cytokines stimulate endothelial expression of CD40 (Noelle et al 1992); iii) recipient endothelial CD40 is crucial for acute allograft rejection (Foy et al 1996); iv) interference with CD40:CD40 ligand interactions inhibits alloreactivity and prolongs allograft survival (Durie et al 1994a; Larsen et al 1996a; Blazar et al 1997; Kirk et al 1997, 2001a; Niimi et al 1998). CD40 ligand stimulation of endothelial CD40 by infiltrating lymphocytes or activated platelets induces expression of adhesion molecules (E-selectin, ICAM-1 and VCAM-1), cytokines/chemokines (IL-6, IL-8, leukaemia inhibitory factor) and growth factors (granulocyte-macrophage colony-stimulating factor) involved in the recruitment of leukocytes, especially CD4 Th1 cells into sites of inflammation (Karmann et al 1995; Hollenbaugh et al 1995; Reul et al 1997; Henn et al 1998).

Cytokine-induced expression of adhesion molecules on endothelial cells leads to the development of endotheliitis, a histological hallmark of rejection, which is characterised by lymphocytic adhesion on the luminal surface, transendothelial migration and ultimately peri-endothelial accumulations of lymphocytes.

6.2. The role of chemokines and chemokine receptors in allograft rejection

Chemokines are chemoattractant proteins which are classified into four subtypes depending on the spacing of cysteine residues within the amino terminal region: C, CC, CXC and CX3C. Chemokines have a role to play in leukocyte activation, selectin/integrin upregulation, haematopoiesis, angiogenesis and adaptive immunity (Strieter et al 1995; Sallusto et al 1999a, 2000; Murphy et al 2000).

Functionally, chemokines may be divided into those expressed constitutively in secondary lymphoid organs and those induced at sites of tissue inflammation. The first group of chemokines, the lymphoid chemokines, are critical for attracting naïve lymphocytes into secondary lymphoid organs, where they are activated by antigenic peptides presented by dendritic cells (Cyster 1999). The lymphoid chemokines are important for immune surveillance (Sallusto et al 2000). The inflammatory chemokines function to recruit neutrophils, monocytes, immature dendritic cells and lymphocytes during tissue inflammation. The inflammatory chemokines are regulated by pro-inflammatory stimuli, e.g. IL-1, TNF-α, LPS and regulate innate and adaptive immune responses. Chemokines mediate their activities through seven transmembrane spanning, G-protein-coupled receptors differentially expressed on leukocyte populations and somatic tissues (Murphy et al 2000; El-Sawy et al 2002). Chemokine receptors not only bind to specific chemokines, but their expression is also regulated according to cell subsets (e.g. Th1 cells express CCR5 and CXCR3 and Th2 cells express CCR3 and CCR4) and the state of cell activation (e.g. immature DCs express CCR1-6, but mature DCs downregulate these six receptors and upregulate CCR7 expression (Sallusto 1999a,b; Sozzani et al 2000; Colvin & Thomson 2002). The CC and CXC chemokines bind to 2 or more receptors, but the C and CX3C chemokines bind to only one receptor. The chemokine receptors can bind to more than one chemokine (Murphy et al 2000; Nelson & Krensky 2001). Chemokines bind to heparin sulphate proteoglycans and glycosaminoglycans on endothelial cells and tissue matrix and are thus able to induce directional transendothelial migration of leukocytes.

In organ transplantation, ischaemia-reperfusion plays an important role in chemokine-mediated recruitment and activation of non-specific and specific effector leukocytes leading to innate immune activation and further propagation of allospecific responses. In transplanted organs, chemokine receptors are expressed on a variety of infiltrating leukocytes that follow the expression and secretion of corresponding chemokines by endothelial, epithelial and stromal cells. Chemokines play a role in antigen-nonspecific graft injury (Nelson & Krensky 2001; El-Sawy et al 2002).

CD4 Th1 and Th2 subsets express different types of chemokine receptors and the endothelial expression of chemokines contributes to the selective migration of T cell subsets. Chemokine expression by endothelial cells stimulated by ischaemia-reperfusion

injury and cytokines also plays a role in selective recruitment of T cell subsets in allograft rejection (Nadeau et al 1995; Kondo et al 1996, 1997; Fairchild et al 1997; Nagano et al 1997). Chemokines within the micro-environment are also able to promote T cell-endothelial cell interaction. Binding of the chemokine stromal cell-derived factor 1 to endothelial cells enhances leukocyte adhesion to ICAM-1 expressed on endothelium (Campbell et al 1998). Following cytokine activation, endothelial cells also express chemokine receptors, especially CXCR4 that are able to bind with stromal cell-derived factor 1 and may promote angiogenesis (Gupta et al 1998).

Chemokines play a critical role in the recruitment of leukocytes into transplanted organs (Melter et al 1999; Nelson & Krensky 2001; El-Sawy et al 2002; Colvin & Thomson 2002). Two general cascades of chemokine production occur during acute rejection (Morita et al 2001). Chemokines are expressed very early following transplantation (within 3 – 72 hours) and are part of the innate immune response to tissue injury occurring during surgery that is directed at the recruitment of neutrophils, macrophages and NK cells. Within minutes following organ reperfusion, pro-inflammatory cytokines including TNF-α and IL-1 are produced by graft endothelial and parenchymal cells. These cytokines stimulate the vascular endothelium and graft parenchymal cells to produce neutrophil- and macrophage-attractant chemokines including IL-8/CXCL8, Gro-α, MIP-2 and MCP-1/CCL2 at early times post transplant. TNF-α and IL-1 also induce upregulated adhesion molecule and MHC molecule expression on vascular endothelium (Bergese et al 1995; Dragun et al 2001). The neutrophil chemoattractants Gro-α and MIP-2 are produced at high levels within 3 hours of reperfusion resulting in rapid neutrophil infiltration (Laskowski et al 2000; Miura et al 2001a). This is followed by the production of chemokines directing the recruitment of macrophages, NK cells and T cells, i.e. MCP-1/CCL2 and macrophage inflammatory proteins (MIP-1α/CCL3 and MIP-1β/CCL4), but at lower levels (El-Sawy et al 2002).

IP-10 (IFN-γ inducible protein), a potent attractant of antigen-activated T cells is induced by IFN-γ and TNF-α (Narumi et al 2000) and may be an important factor mediating transition from early non-specific infiltration to allo-antigen-specific graft infiltration. MIP-1α preferentially recruits CD8 T cells, whereas MIP-1β preferentially recruits CD4 T cells (Krakauer et al 1999). As the initial inflammatory response subsides, a second wave of intragraft chemokine expression occurs. These later

chemokines (48-72 hours+) include MIG (monokine induced by IFNγ/CXCL9), IP-10/CXCL10, I-TAC and RANTES (regulated upon activation normal T cell expressed and secreted) (Fairchild 1998). The expression of these late chemokines is dependent on the presence of T cells within the allograft and is part of the adaptive immune response. The production of the chemokines, except for IP-10, is dependent on locally produced IFN-γ. IP-10 attracts resting and activated T cells, NK cells and monocytes (Krakauer et al 1999). MIG attracts resting and activated T cells, whilst RANTES primarily recruits memory CD4 T cells, monocytes and eosinophils into the allograft (Krakauer et al 1999).

Studies suggest that circulating memory-phenotype CD8 T cells that are not previously primed to graft alloantigen are directed to sites of neutrophil-mediated inflammatory foci in the vascular endothelium of allografts and T cells with reactivity to allogeneic class I are stimulated to produce IFN-γ. IFN-γ binding to IFN-γ receptors on the endothelial cells stimulates production of MIG by the endothelium. IFN-γ, IL-8 and other chemokines bind to proteoglycans on the endothelial cell surface. As neutrophils and macrophages arrest on the endothelial surface, their IFN-γ receptors bind the immobilised IFN-γ and this stimulates the leukocyte to produce MIG and IP-10 as they infiltrate the allograft parenchyma (Miura et al 2001b; Kapoor et al 2000; El-Sawy et al 2002).

Dendritic cell/chemokine interactions play an important role in allograft rejection. Immature dendritic cells migrate to sites of inflammation in response to the inducible chemokines (CCL-2-5) released by activated endothelial cells (Sozzoni et al 2000; Caux et al 2000) and mature dendritic cells are recruited to secondary lymphoid tissues by constitutively expressed chemokines, CCL19 and CCL21 (Colvin & Thomson 2002). Chemokines secreted by dendritic cells are involved in T cell recruitment (Colvin & Thomson 2002).

Chemokines may also play a role in chronic allograft rejection. RANTES (CCL5) production has been found to correlate with leukocyte infiltration as well as preceding intimal thickening of arterial vessels in cardiac allograft vasculopathy (Yun et al 2001).

Different chemokines may vary in their importance in determining allograft survival depending on the type of allograft (Colvin & Thomson 2002). Analysis of chemokine

gene expression during allograft rejection has shown that CXCL5 and CCL2 expression was independent of T cell infiltration, whilst intragraft expression of CCL3, CCL4, CCL5, CXCL9, CXCL10, XCL1 and CCL1 was T cell dependent and increased with time after transplantation (Carvalho-Gaspar et al 2005). Studies have shown that combination therapies blocking chemokine/chemokine receptor expression together with administration of immunosuppressive agents (e.g. Cyclosporin A in CCR1 and CXCR3 KO models) can result in indefinite graft survival (Gao et al 2000; Hancock et al 2000; Colvin & Thomson 2002). Combined CXCR3 and CCR5 blockade has been shown to prolong cardiac allograft survival in a fully MHC mismatched murine model (Schnickel et al 2006). Combined blockade of CCR1 and CCR5 attenuated the development of cardiac allograft vasculopathy (Yun et al 2004).

6.3. The role of cytokines in acute allograft rejection

Cytokines are produced by a variety of cells (including lymphocytes, monocytes, dendritic cells and stromal cells) involved in the immune response (Abbas et al 1997). Cytokines are involved not only in the process of acute rejection of transplanted organs, but also in the induction of immunological tolerance. Professional antigen-presenting cells, mainly dendritic cells, are essential for initiating the alloimmune response. These cells allow for specific activation of T lymphocytes by engagement of the T cell receptor and provide co-stimulatory signals. Once activated, alloreactive T lymphocytes proliferate and differentiate into effector and helper cells. The majority of helper T cells are of the CD4 phenotype, whilst effector T lymphocytes such as cytotoxic T lymphocytes (CTL) are mainly $CD8^+$. The differentiation of both $CD4^+$ and $CD8^+$ T cells is influenced by cytokines produced by dendritic cells. Interleukin 12 which is secreted by activated dendritic cells facilitates differentiation of $CD8^+$ T cells into CTL and the differentiation of $CD4^+$ T cells into the pro-inflammatory Th1 phenotype (Lane & Brocker 1999). The Th1-derived cytokines include IL-2, IL-3, IFN-γ and TNFα and promote the immunopathological reactions leading to allograft injury.

Cytokine pathways, however, are highly redundant, as has been shown in acute allograft rejection experiments performed in cytokine gene-knockout mice. Despite the essential role of IL-2 as an *in vitro* T cell mitogen, IL-2 gene-knockout (IL-2 $^{-/-}$) mice are capable of rejecting pancreatic islet and vascularised cardiac allografts and generate normal

numbers of allospecific CTLs (Steiger et al 1995; Dai et al 1998). Double gene-knockout mice deficient in both IL-2 and IL-4 reject fully allogeneic pancreatic islet cells as vigorously as wild-type recipients, suggesting that other T cell mitogens whose receptors share the common cytokine receptor gamma chain (γc), such as IL-7, IL-9 and IL-15 (Alves et al 2007), mediate T cell proliferation in the absence of IL-2 or IL-4 (Li et al 1998). Antibodies which target γc significantly prolong pancreatic islet allograft survival in mice (Chang et al 2000). Interferon-γ has been shown *in vitro* to be a potent monocyte and T cell activator, yet the absence of interferon-γ or its receptor in recipient mice does not delay the acute rejection of fully allogeneic cardiac or pancreatic islet grafts (Saleem et al 1996; Steiger et al 1998). Acute cardiac allograft rejection may be accelerated in IFNγ$^{-/-}$ double-knockout mice (Saleem et al 1996). Antagonising IL-12, despite inducing Th2 cytokine expression within the allograft, accelerated cardiac allograft rejection and this was associated with allograft IFN-γ gene expression (Picotti et al 1996). Thus, although each cytokine may contribute to the rejection process, no single cytokine is necessary for rejection to occur. An exception to cytokine redundancy is the finding that IFN-γ is essential for acute rejection of MHC class II incompatible allografts. It is thought that IFN-γ is essential for the initiation of alloimmune responses to MHC class II antigens by upregulating MHC class II expression on antigen-presenting cells and endothelial cells (Goes et al 1995, 1996; Ring et al 1999).

7. REGULATION OF THE ALLOIMMUNE RESPONSE

Feedback immune regulatory mechanisms which downregulate activated T lymphocytes, are essential for keeping immune responses in check and for maintaining tolerance to self-antigens (van Parijs & Abbas 1998). The regulation of an activated T cell population leads to a decrease in effector function and eventual disappearance of activated T lymphocytes, leaving long-lived functionally quiescent memory lymphocytes as the only surviving indicators of previous antigen exposure.

Intrinsic mechanisms that terminate adaptive immune responses and restore the quiescent state of activated alloreactive T cells include deletion (van Parijs & Abbas 1998; Wells et al 1999), anergy (Schwartz 2003), clonal exhaustion (Bishop et al 1997) and ignorance (Chalasani & Lakkis 2001). In addition, as previously described,

alloreactive T cells also receive suppressive signals from regulatory T cells (Wood & Sakaguchi 2003; Boschiero et al 2007).

Activated T cells are shortlived as peripheral deletion mechanisms operate to delete specificities from the proliferating lymphocyte repertoires. As the antigenic stimuli and the innate immune reaction subsides, lymphocytes fail to acquire signals from survival factors and passive cell death (PCD) is triggered as a result of reduced expression of members of the anti-apoptotic Bcl family (van Parijs & Abbas 1998; Li et al 2001a). IL-2 via IL-2Rα and gamma chain signaling induces not only T cell proliferation and survival, but also sensitizes cells to apoptosis (activation-induced cell death, AICD) via the Fas/Fas ligand pathway, a feature that is essential for tolerance induction and is not shared by other T cell growth factors (Marrack et al 2000; Li et al 2001a; Khaled & Durum 2002; Alves et al 2007). AICD as a result of continued antigenic stimulation also leads to death of neighbouring T cells. Interventions that promote PCD via the IL-15Fc or AICD via the IL-2 Fc induce indefinite allograft survival in mice (Li et al 2001b). At the costimulatory level, CTLA-4 and a number of other novel negative costimulatory molecules exert a negative feedback loop, thereby inhibiting IL-2 synthesis and progression through the cell cycle (Alegre et al 2001; Sharpe & Freeman 2002; Greenwald et al 2002, 2005).

In clinical transplantation, longterm regulation of the allo-immune response and tolerance induction is difficult as the transplanted organ is not only highly antigenic, but a persistent source of alloantigens and there is marked redundancy of activation pathways necessitating the need to target multiple pathways.

CHAPTER FOUR:

LITERATURE REVIEW : IMMUNOSUPPRESSIVE AGENTS

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CHAPTER FOUR:

LITERATURE REVIEW : IMMUNOSUPPRESSIVE AGENTS

1. INTRODUCTION

T cells play a central role in the specific immune response of an acute allograft rejection. The generation of the immune response to most antigens is critically dependent on the initial phase of T cell activation and proliferation. Immunosuppressive regimens have looked at preventing T cell activation or effector function. Prior to interaction with the antigen-presenting cell and recognition of the alloantigen, the mature resting T cell is in the G0 phase of the cell cycle. Following contact with the antigen-presenting cell and binding of the alloantigen presented in the context of the MHC molecule to the T cell receptor (TCR) on the T lymphocyte surface through either direct or indirect pathways, T cell activation is initiated, resulting in generation of signals required for cell cycle entry (G0 – G1 phase transition) (Colvin 1990; Sayegh et al 1994) and the expression of high affinity receptors for T-cell growth factors including IL-2 and IL-4.

Following engagement of the TCR with the alloantigens, a series of tyrosine kinases including Lck, Fyn and ZAP-70 are recruited and activated, resulting in phosphorylation and activation of phospholipase C and a rise in intracellular calcium (Colvin 1990). The rise in intracellular calcium leads to activation of calcineurin, a serine-threonine phosphatase that transduces signals to the nucleus to transcribe genes encoding cytokines that are required for the transition of the T cell from the resting to the activated state. In order to sustain full T cell activation, additional co-stimulatory signals need to be provided by the antigen-presenting cells (Steinman & Young 1991). The co-stimulatory signals driven by CD28, synergise with TCR stimulation, allowing transcription of genes for cytokines that induce T cells to move from the resting G0 phase to the activated G1 state (Schwartz et al 1989; Schwartz 2003; Appleman & Boussiotis 2003). The progression from G1 to the S phase is regulated through the generation of growth receptors such as the IL-2 receptor expressed on the T cell surface

and all acting on common intracellular elements in a pathway that controls enzymes such as the cyclin/cyclin-dependent kinases that are important in the induction of cell division. De novo purine and pyrimidine synthesis is required in order for lymphocytes to complete cell division. Immunosuppressive agents interacting at different steps in these pathways help prevent T-cell activation and proliferation and control graft rejection.

At present, the standard immunosuppressant regimens include 5 drug classes:

1. Calcineurin inhibitors – Cyclosporin, FK506 (Tacrolimus).
2. Antimetabolites – azathioprine, mycophenolate mofetil
3. Corticosteroids
4. mTOR inhibitors – Rapamycin (Sirolimus)
5. Anti-T cell antibodies – ATG (antithymocyte globulin), anti-CD3 antibody (OKT3), IL-2 receptor antibody

At present, costimulatory blockade, as discussed in Chapter 5, is not in routine practice.

Immunosuppressive agents act as pharmacological probes of lymphocyte signal transduction as characterisation of the biochemical processes altered by immunosuppressives, yields important information regarding signal transduction pathways involved in T-cell activation. They also provide insight into the potential development of novel immunosuppressants.

The calcineurin inhibitors (Cyclosporin and FK506/Tacrolimus) and the mMTOR inhibitor, Rapamycin, will be discussed in this chapter.

2. CALCINEURIN INHIBITORS

2.1. Cyclosporin and FK506 (Tacrolimus)

Cyclosporin and FK506 are both microbial products with potent immunosuppressant properties resulting in a selective inhibition of T cell activation. Cyclosporin was isolated from cultures of the fungus *Tolypocladium inflatum gams* from a soil sample collected in Southern Norway (Petcher et al 1976), whilst FK506 ***(***Tacrolimus***)*** (Kino et al 1987a) was isolated in the fermentation broth of *Streptomyces tsukubaensis*, a soil micro-organism found in soil samples collected in the Tsukuba area of Northern Japan. Cyclosporin is a cyclic undecapeptide with hydrophobic characteristics due to N-methylation of 7 of the 11 peptide nitrogens and shielding of the hydrophilic side chains at positions 11, 1 and 2 in the internal side of the molecule (Mason 1992). FK506 is a macrolide lactone with a hemiketal-masked α, β - diketoamide incorporated into a 23 member ring (Tanaka et al 1987a,b). Although chemically unrelated, Cyclosporin and FK506 both inhibit similar subsets of calcium-dependent activation pathways (Lin et al 1991) involved in the regulation of lymphokine gene expression, activation-driven T cell death and exocytosis. Cyclosporin and FK506 both exert their inhibitory effect during the G_0 to G_1 phase of T cell activation and block the expression of the same set of early lymphokine genes (Tocci et al 1989; Metcalfe & Richards 1990).

2.1.1. Biochemical targets and mechanisms of action of calcineurin inhibitors

Cyclosporin and FK506 only become biologically active, when complexed with distinct endogenous intracellular receptors (cytosolic binding proteins) known as immunophilins. These intracellular receptors are abundant, ubiquitous and phylogenetically well conserved (Koletsky et al 1986; Siekierka et al 1990). The major cytosolic receptor for Cyclosporin is cyclophilin A, a 18 kDa protein (Harding et al 1986), whilst the major FK506 binding protein (FKBP) is a 12 kDa cytosolic protein named FKBP12 (Siekierka et al 1989; Harding et al 1989). Cycophilin A (Takahashi et al 1989; Fischer et al 1989) and FKBP12 (Siekierka et al 1989; Harding et al 1989) are peptidyl-prolyl isomerases (PPIases), which catalyse the *cis-trans* isomerisation of peptidyl-prolyl bonds in peptides and proteins. FKPB12 prefers substrates with hydrophobic amino acids immediately preceding the proline, in contrast to cycophilin A

which is a more promiscuous enzyme (Harrison & Stein 1990). Although, Cyclosporin (Takahashi 1989; Fischer et al 1989) and FK506 (Siekierka et al 1989; Harding et al 1989) both act as inhibitors of the PPIase activity of their respective cytosolic binding proteins, the inhibition of the PPIase activity is unrelated to their immunusuppressive properties (Bierer et al 1990b; Sigal et al 1991; Dumont et al 1994). The formation of the immunophilin-drug complexes is essential for the biological activity of Cyclosporin and FK506, in a gain-of-function model. These active complexes interfere with intracellular calcium-dependent signal transduction pathways, processes that are central to T cell activation (Bierer et al 1990a, 1990b; De Franco 1991; Schreiber 1991; Schreiber & Crabtree 1992). The common biological target for these drug-immunophilin complexes is the calcium- and calmodulin-dependent serine-threonine phosphatase, calcineurin (Liu et al 1991; Fruman et al 1992; Schreiber & Crabtree 1992), a critical, rate-limiting component of the TCR-linked signal transduction pathway leading to cytokine gene transcription (O'Keefe et al 1992; Clipstone & Crabtree 1992; Shibasaki et al 2002).

The signal transduction cascade begins with antigen presentation to the T cell receptor which leads to an increase in intracellular calcium (Liu 1993) and a subsequent activation of the calmodulin-calcineurin complex. The nuclear factor of activated T cell (NF-AT) plays an essential role in conveying the signal from the cytoplasm to the nucleus in order to activate transcription of the IL-2 genes (Shaw et al 1988; Sigal & Dumont 1992; McCaffrey et al 1993). This T-cell specific transcription factor has two subunits, one of which is phosphorylated and thereby confined to the cytoplasm, while the other is predominantly nuclear (Flanagan et al 1991). IL-2 gene transcription can only take place once the nuclear and cytoplasmic NF-AT subunits are combined. An increase in intracellular calcium activates calcineurin phosphatase, which then dephosphorylates the cytoplasmic NF-AT. The dephosphorylated cytoplasmic NF-AT subunit is then free to translocate to the nucleus and associate with the nuclear subunit, thus forming a fully active NF-AT complex, an essential component of the transcriptional apparatus required for the expression of IL-2 and other pro-inflammatory cytokine genes such as IL-3, IL-4, IFN-γ and TNF (Flanagan et al 1991). The immunophilin-drug complexes of Cyclosporin and FK506 inhibit the transcriptional activity of NF-AT by preventing the assembly of a functional transcription factor (Flanagan et al 1991; Timmerman et al 1996). Both Cyclosporin and FK506 in the form

of a drug-immunophilin complex interfere with the TCR-mediated activation of calcineurin, thereby blocking transcription of the IL-2 gene in T lymphocytes (O'Keefe et al 1992; Sigal & Dumont 1992; Fruman et al 1992; Siekierka 1994; Crabtree & Clipstone 1994).

In addition, both Cyclosporin and FK506 block the activation of JNK and p38 pathways, but not the ERK pathway during T cell activation. This inhibition occurs at a level upstream of mitogen-activated protein kinase (MAPK) kinase kinase (MAPKK-K) and is independent of calcineurin inhibition (Matsuda et al 2000). The inhibition of both the calcineurin-dependent NF-AT pathway and the calcineurin –independent JNK and p38 activation pathways leads to impaired IL-2 gene transcription (Matsuda at al 1998, 2000). Since IL-2 plays a critical role as a growth and regulatory factor for T cells, inhibition of IL-2 production is one of the principal mechanisms of immunosuppression by FK506 and Cyclosporin.

Cyclosporin has been shown to affect the lymphocyte plasma membrane causing early depolarisation (Damjanovich et al 1987) and transient rigidification (Niebylski et al 1991). Cyclosporin has also been shown to increase the production of lysophospholipid and thereby inhibit membrane-associated Na^+K^+-ATPase which is upregulated during T cell activation (Anderson et al 1993).

2.1.2. Effect on T lymphocyte proliferation

FK506 and Cyclosporin inhibit calcium-dependent T cell activation pathways triggered by the T cell receptor/CD3 complex (Kay et al 1989a,b; Johansson & Möller 1990; Lin et al 1991; Bierer et al 1991a; Jiang et al 1991), the cell surface CD2 receptor (Kay & Benzie 1989; Bierer et al 1991b) and the combination of protein kinase C activation and calcium influx (Kay et al 1989b; Dumont et al 1990a, 1990b).

This has been demonstrated using in vitro models of murine and human T cell proliferation in response to a variety of the stimuli. Both Cyclosporin and FK506 have been shown to inhibit T cell proliferation in response to mitogenic lectins such as Concanavalin A (Con A) and phytohaemagglutinin A (PHA); monoclonal antibodies against the T cell receptor/CD3 complex and other cell surface receptors and the combination of calcium ionophore (ionomycin) with the phorbol ester PMA (Kay et al

1989a,b, 1990; Bloemena et al 1989; Dumont et al 1990b; Johansson and Möller 1990; Bierer et al 1991a)). Cyclosporin and FK506 also suppress proliferation of alloreactive cells in mixed lymphocyte reactions (MLR) (Hess & Tutschka 1980; Kino et al 1987b; Yoshimura et al 1989a; Thomas et al 1990), as well as inhibit antigen-specific proliferation of cloned helper and cytotoxic T-cells (Herold et al 1986; Sawada et al 1987; Havele & Paetkau 1988). FK506 is approximately 30-100 times more potent than Cyclosporin *in vitro* in inhibiting T cell proliferative responses including mixed lymphocyte reactions and cytotoxic T-cell generation (Kino et al 1987b; Sawada et al 1987), exhibiting an IC50 of approximately 0.2 nM – 0.5 nM as compared to the IC50 of 10 to 100 nM seen with Cyclosporin (Sigal & Dumont 1992). In addition, in the concentration range similar to that which inhibits cell division, FK506 has also been shown to inhibit the generation of cytotoxic T cells specific for allogeneic targets. *In vitro* cellular cytotoxicity mediated by NK cells and killer cells and antibody-dependent cell-mediated cytotoxicity is unaffected by FK506 (Beck & Akiyama 1989; Markus et al 1991; Wasik et al 1991; Alamartine et al 1994). Cyclosporin also fails to inhibit alloreactive NK cell proliferation and cytotoxicity (Lefkowitz et al 1988; Kosugi & Shearer 1991; Petersson et al 1997).

In a human MLR model, Cyclosporin and FK506 inhibited induced FOXP3 gene transcription (Baan et al 2005). Cyclosporin inhibits expansion of $CD4^+$ $CD25^+$ Tregs following alloantigen stimulation (Lim et al 2007) as well as inhibiting de novo FOXP3 expression in activated murine $CD4^+$ $FOXP3^-$ T cells in vitro (Gao et al 2007).

Cyclosporin inhibited the generation of $CD4^+$ $CD25^+$ CTLA-4^+ and $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs in renal transplant recipients (Korczak-Kowalska et al 2007). Cyclosporin inhibits the differentiation of both $FOXP3^+$ Tregs and CD4 Th17 cells (Kopf et al 2007). Cyclosporin and Tacrolimus do not alter the generation of $CD103^+$ $CD8^+$ regulatory T cells following alloantigen stimulation (Uss et al 2007).

However, even at μM concentrations, Cyclosporin and FK506 have been shown to have little effect on the proliferative responses induced by growth-promoting cytokines such as IL-2 or IL-4 (Hess et al 1982; Kino et al 1987b, Yoshimura 1989b; Kay et al 1989b; Dumont et al 1990a,b). Cyclosporin and FK506 have no effect on calcium-independent T cell activation such as that triggered by the CD28 surface molecule or protein kinase C activation alone (June et al 1987; Kay & Benzie 1989; Thompson et al 1989; Bierer et

al 1991a; Chang et al 1991; Lin et al 1991). The calcium-independent CD28 pathway is an alternative pathway of the T cell activation, which is triggered by the interaction of the CD28 molecule on T cells with its specific ligands B7-1 and B7-2 expressed on monocytes/macrophages and activated B cells and serves as a co-stimulatory signal for the TCR/CD3 pathway resulting in markedly enhanced IL-2 production (Fraser et al 1991).

Both Cyclosporin and FK506 act early in T cell signal transduction, blocking calcium-dependent T-cell division between the resting phase G0 and activation phase G1 of the cell cycle and suppressing the transcription of early phase cytokine genes (Tocci et al 1989; Metcalfe & Richards 1990; Chang et al 1991; Morris et al 1991). As a result, the anti-proliferative effect is only observed, when both drugs are added within the first few hours of T cell stimulation (Hess & Tutschka 1980; Kay & Benzie 1984; Tocci et al 1989; Kay et al 1989b, 1990; Dumont et al 1990b; Metcalfe & Richards 1990; Henderson et al 1991). The inhibitory activity of FK506 and Cyclosporin on T-cell proliferation is only partially reversed by exogenously added IL-2 (Hess 1985; Gelfand et al 1987; Dumont et al 1990b; Lin et al 1991), suggesting that both agents are effective only when endogenous IL-2 is involved in T lymphocyte growth and that other gene products and pathways critical for cell cycle progression remain intact.

2.1.3. Effect on cytokine gene expression and production

T cell activation is initiated by the recognition of foreign antigen via the TCR/CD3 complex. The TCR/CD3 mediated signaling pathway simultaneously requires the help of cytokines such as IL-1 and IL-6 that are produced by antigen-presenting cells (Weaver & Unanue 1990). These signals produced at the cell membrane are transferred into the nucleus of T lymphocyte through a complex series of biochemical reactions (Nel 2002). FK506 (Kay et al 1989a,b) and Cyclosporin (Bijsterbosch & Klaus 1985) do not affect the early events in these processes such as the generation of the intracellular second messengers, inositol triphosphate (IP3) and diacylglycerol (DAG), which result in the increase in intracellular concentration of calcium and activation of protein kinase C (PKC) respectively and the subsequent phosphorylation of some cytoplasmic proteins (Fidelus & Laughter 1986; Fujii et al 1989; Bierer et al 1990b; Jordan et al 1991). Following these events, the expression of a set of gene products

crucial for T cell activation and proliferation is eventually induced. Both FK506 and Cyclosporin inhibit these more distal components of the T cell activation pathway. Cyclosporin has been shown to inhibit the expression of IL-2 gene in heterogenous T cell populations (Kronke et al 1984; Granelli-Piperno 1988a, 1990) as well as in cloned T-cell lines (Herold et al 1986). FK506 has been shown to inhibit the transcription of mRNA for several cytokines including IL-2, IL-3, IL-4, GM-CSF, TNF-α, and interferon γ (Tocci et al 1989) at an *in vitro* concentration 10 to 100 times less than that for Cyclosporin. Both FK506 and Cyclosporin have been shown *in vitro* to inhibit TNF-α and IL-Iβ production by PBMCs stimulated with immobilised anti-CD3/CD28 monoclonal antibody, but FK506 was a more potent inhibitor (Sakuma et al 2000). FK506 is similarly more potent than Cyclosporin in inhibiting transcription of proto-oncogenes such as ras, myc and rel as well as suppressing the IL-2 and IL-7 receptor expression (Peters et al 1993; Morris 1993, 1996; Manez et al 1995; Thomson et al 1995; De Mattos et al 1996). FK506 and Cyclosporin both preferentially suppress cytokine production stimulated by Th1 cells (such as IL-2, TNF-β and interferon-γ which are important in cell-mediated immunity) over cytokines produced by Th2 cells such as IL-4, IL-5 and IL-10 which are more involved in B cell stimulation and antibody production (Thomson et al 1995).

FK506 (Bierer et al 1991a) and Cyclosporin (Thompson et al 1989) do not inhibit IL-2 production in the calcium-independent CD28 pathway, an alternative pathway for T cell activation. FK506 and Cyclosporin only inhibit activation events which result in a notable rise in intracellular calcium concentration.

FK506 and Cyclosporin directly interfere with the transcription of the IL-2 gene (Bunjes et al 1981; Dos Reis & Shevach 1982; Kronke et al 1984; Granelli-Piperno et al 1984; Tocci et al 1989). The IL-2 gene is silent in resting T cells, but is activated after antigen recognition by the specific TCR/CDR complex and subsequent signal transduction into the nucleus. As discussed above, the expression of the IL-2 gene is controlled by the interaction of specific nuclear transcription factors with their corresponding DNA sites on the IL-2 gene enhancer/promoter (Fujita et al 1986; Durand et al 1988; Crabtree 1989). Several transcription factors including NF-AT, NF-κB, AP-1 and Oct-1 are involved in regulation of IL-2 gene expression. NF-AT (nuclear factor of activated T cells) is unique, because its expression is restricted to activated T

cells (Shaw et al 1988), whilst the others are commonly found in almost all cells and tissues and are responsible for regulation of the transcription of various genes. Cyclosporin and FK506 specifically inhibit the binding of NF-AT to its binding site on the IL-2 enhancer, have no effect on AP-1 binding activity and the binding of NF-κB and Oct-1 is only marginally affected (Emmel et al 1989; Granelli-Piperno et al 1990; Mattila et al 1990). The highly restricted distribution of the cytoplasmic NF-AT may account for the selective action of FK506 and Cyclosporin on T cells. Both Cyclosporin and FK506 inhibit calcium-dependent activation of NF-κB multimers which requires new protein synthesis, but protein synthesis-independent induction of NF-κB by PMA is not suppressed (Mattila et al 1990).

Cyclosporin does not inhibit the transcriptional initiation of the IL-2 receptor (IL-2R) gene (Reed et al 1986; Tocci et al 1989), but in vitro studies have shown that IL-2R expression is variably inhibited by Cyclosporin and Tacrolimus depending on the stimulus and duration of culture (Lillehoj et al 1984; Ryffel et al 1995; Dumont et al 1990b; Foxwell et al 1990; Woo et al 1990c) and can be reversed by the addition of exogenous IL-2.

Activation of T-cells with ionomycin plus PMA results in the rapid disappearance of ets-1 mRNA which is normally expressed constitutively by mature T cells (Bhat et al 1990). FK506 has been shown to block this downregulation, thereby restoring ets-1 mRNA expression to a resting level (Dumont et al 1991).

2.1.4. Other immunosuppressive effects

FK506 and Cyclosporin do not inhibit T cell adhesion (Eiras et al 1991), but FK506 inhibits migration of CD4 and CD8 T cells in response to various chemotactic factors (Adams & Liu 1996). FK506 and Cyclosporin have been shown to inhibit various types of exocytosis-related events in both lymphocytes and non-lymphocytes including TCR-mediated degranulation of cytotoxic T cells (Dutz et al 1993), calcium ionophore-induced degranulation of neutrophils and basophils (Forrest et al 1991; De Paulis et al 1992) and IgE receptor mediated histamine and serotonin release from mast cells and basophils (Hultsch et al 1991; De Paulis et al 1992).

Cyclosporin and FK506 have been shown to inhibit the release of granule-associated serine esterase from murine cytotoxic lymphocytes triggered with anti-CD3 or by the combination of calcium ionophore and PMA (Lancki et al 1989; Trenn et al 1989; Dutz et al 1993). Thus, both Cyclosporin and FK506 interfere with calcium-dependent cytoplasmic events that control the vectorial translocation of secretory granules across the plasma membrane.

Cyclosporin blocks activation induced cell death (AICD) of peripheral T cells (Wells et al 1999; Li et al 1999; Strauss et al 2002; Takahashi et al 2004). FK506 has been shown to inhibit activation induced cell death (Strauss et al 2002; Takahashi et al 2004) as well as augment activation induced cell death of peripheral T cells (Migita et al 1995). Activation induced cell death is required for the generation and maintenance of self-tolerance and therefore calcineurin inhibitors may interfere with the potential to develop allograft tolerance.

Cyclosporin has been shown to induce increased PGE production by human monocytes. This increased PGE production was sensitive to indomethacin, a non-selective cyclooxygenase inhibitor, but Cyclosporin also increased the availability of free arachidonic acid (Whisler et al 1984).

2.1.5. Effect on B lymphocytes

Cyclosporin and FK506 inhibit the proliferative response of purified murine and human B cells induced by stimuli which result in the rapid increase in intracellular calcium levels including anti-IgM antibodies, *Staphylococcus aureas* Cowan strain 1 (SAC) or phorbol ester plus calcium ionophore at concentrations that inhibit T-cell responses (Klaus 1988; Walliser et al 1989; Wicker et al 1990; Morikawa et al 1992), whereas calcium-independent lipopolysaccharide-induced B cell proliferation is not affected by FK506 (Walliser et al 1989). *In vitro*, FK506 inhibits IgM and IgG production by mitogen-stimulated human B lymphocytes, but does not inhibit IL-6 production (Yoshimura et al 1989b) or IL-6-induced IgM and IgG production (Yoshimura et al 1989b; Stevens et al 1991). *In vivo*, FK506 suppresses T-cell dependent IgM production by murine and rat splenic plasma cells (Kino et al 1987a,b; Woo et al 1988, 1990b; Tsuji et al 1990; Lagodzinski et al 1991). These effects may be attributed in part to inhibition of cytokine production by activated T cells (Woo et al 1988; Suzuki et al

1990), but FK506 also has a direct inhibitory effect on calcium-dependent B cell activation. FK506 and Cyclosporin block induction of the TNF-α gene transcription by anti-Ig antibody in human B cells (Goldfeld et al 1992). FK506 also appears to inhibit human B cell proliferation responses to certain calcium-independent stimuli including the protein kinase C activator PMA and IL-2 (Morikawa et al 1992). FK506 and Cyclosporin block B cell division in the late activation G1 phase of the cell cycle and as a result, inhibition of B cell proliferation is evident, even when the drug is added as late as 24 hours following the stimulation with anti-IgG (Muraguchi et al 1983; Wicker et al 1990). FK506 causes cell death upon activation of murine B cells (Wicker et al 1990).

2.1.6. Effect on non-lymphoid cellular activation

Cyclosporin and FK506 do not affect IL-1 mRNA expression or production by macrophages (Granelli-Piperno 1988b; Tocci et al 1989). Low doses of Cyclosporin have been reported to inhibit LPS-induced TNF-α bio-activity in the supernatant of macrophage cultures, but do not block expression of TNF-α mRNA, thus suggesting that Cyclosporin may affect a post-transcriptional step of TNA-α biosynthesis in macrophages (Nguyen et al 1990). FK506 does not interfere with antigen presentation nor modify mononuclear phagocyte function (Peters et al 1993; Manez et al 1995; Thomson et al 1995; Morris 1996). Cyclosporin has been shown to inhibit alloantigen presentation by murine macrophages without affecting the expression of MHC class II molecules (Little et al 1990). The doses of Cyclosporin required to suppress antigen presentation are much higher than those required to suppress cytokine production. The concentration of FK506 which results in significant inhibition of soluble antigen-induced human T-cell proliferation does not significantly impair the accessory function of mononuclear phagocytes (Woo et al 1990a) and thus direct inhibition of antigen processing does not appear to be an important factor in the *in vivo* mechanism of action of FK506 and Cyclosporin.

3. mTOR INHIBITORS

3.1. Rapamycin

Rapamycin is a lipophilic macrolide, produced by a strain of *Streptomyces hygroscopicus*, isolated from a soil sample on Easter Island, with both immunosuppressive and anti-proliferative properties (Sehgal et al 1975; Vezina 1975). Unlike Cyclosporin and Tacrolimus, it does not inhibit early phase lymphocyte activation genes, but is a potent inhibitor of cytokine and growth-factor mediated cellular proliferation, thereby preventing cell cycle progression from the G1 to the S phase in the T cells (Terada et al 1993; Sehgal et al 1995; Sehgal 1995, 1998).

3.1.1. Biochemical targets and mechanisms of action of Rapamycin

Rapamycin binds to the immunophilin, FKBP12, but the mechanism of action of rapamycin is distinct from that of Cyclosporin or FK506. The immunosuppressive effects of rapamycin relate to the formation of the rapamycin-FKBP12 complex (Wood & Bierer 1994; Wiederrecht & Etzkorn 1994; Galat & Metcalfe 1995). Although the formation of the rapamycin-FKBP12 complex is essential for rapamycin's biological activity, the inhibition of the immunophilin's PPIase activity is not associated with the drug's immunosuppressive properties (Sehgal et al 1994; Wood & Bierer 1994; Wiederrecht & Etzkorn 1994; Galat & Metcalfe 1995). The formation of the drug-immunophilin complex results in "gain of function" with the complex modulating the activity of other specific intracellular targets.

The rapamycin-FKBP complex binds to a specific cell-cycle regulatory protein called the mammalian target of rapamycin (mTOR) and inhibits its activation. Despite their structural similarity and shared immunophilins, the mechanism of rapamycin is distinct from FK506. FK506, like Cyclosporin, inhibits T cell proliferation in the G_0-G_1 phase of the cell cycle and rapamycin prevents cell cycle progression from G_1-S phase in various cell types (Terada et al 1993; Albers et al 1993; Jayaraman & Marks 1993; Marx et al 1995).

Unlike Tacrolimus and Cyclosporin, the rapamycin-FKBP12 complex does not inhibit calcineurin. The sensitivity of T cells to rapamycin correlates with the binding of the

rapamycin-FKBP12 complex to mTOR (Abraham & Wiederrecht 1996; Abraham 1998).

Like FK506, rapamycin has 2 domains – an effector domain forming a composite surface with FKBP which interacts with mTOR and a binding domain which mediates the interaction with FKBP. The FK-binding domain is conserved in FK506 and rapamycin and both drugs compete for this domain resulting in mutual antagonism (Dumont et al 1990a; Bierer et al 1990a). In mTOR, the binding domain is a 133 hydrophobic amino acid region just upstream of the lipid kinase domain, and contains a critical serine residue (Ser^{2035}) (Chiu et al 1994; Chen et al 1995). The FKBP12-rapamycin complex binds to and inhibits the function of the target protein, mTOR, a serine-threonine kinase with homology to phosphotidyl-inositol 3-kinase (PI-3-kinase). mTOR autophosphorylates its serine residues (Zheng et al 1995).

mTOR plays a critical role in the signal 3 pathway of T cell activation leading to 2 pathways of translational control by mitogenic stimuli – the activation of $p70^{S6}$ kinase pathway and the eIF-4E/PHAS-I pathway (Abraham & Wiederrecht 1996; Abraham 1998; Rowinsky 2004; Jastrzebski et al 2007). mTOR is a downstream mediator of the PI-3-kinase/AKT signaling pathway and plays a central role controlling cellular growth and division (Schmelzle & Hall 2000; Gingas et al 2001; Huang et al 2003; Fingar & Blenis 2004). mTOR, once activated, transduces signals that initiate synthesis of ribosomal proteins, translation of a specific subset of messenger RNA transcripts and generation of cyclin-dependent kinases, promoting the progression of the cell cycle, leading to activation, growth and proliferation of T and B cells and antibody production (Brown & Schreiber 1996; Abraham & Wiederrecht 1996; Abraham 1998; Gummert et al 1999; Rovira et al 2000; Lee & Hung 2007).

Following the calcium-dependent signal 1 pathway and in the presence of the calcium-independent signal 2 pathway, the nuclear factor of activated T-cells (NF-AT) is dephosphorylated after activation of calcineurin. Following transition to the nucleus, NF-AT promotes cytokine and growth factor transcription. These growth factors, including IL-2, IL-4, IL-7, IL-12, IL-15, IFN-γ, platelet-derived growth factor, basic fibroblastic growth factor, transforming growth factor-β and insulin bind in an autocrine or paracrine manner to their specific receptors on the cell surface thereby delivering signal 3. The resulting kinase cascades lead to mTOR activation by

autophosphorylation. T-cell activation via CD28 is also sensitive to rapamycin, suggesting that mTOR activation is also possible through signal 2.

As a result of the binding of the FKBP12-rapamycin complex to its effector protein mTOR, a number of biochemical events essential for cell cycle progression are inhibited, resulting in cell arrest in the late G1 phase just prior to entry into the S phase (Bierer et al 1990a; Morice et al 1993; Terada et al 1993; Sehgal 1995, 1998). The Rapamycin-FKBP12 complex inhibits mTOR and the signal 3 pathway, i.e. G1-S transition, translation and cytokine-driven T-cell proliferation.

Rapamycin inhibits activation of $p70^{S6}$ kinase which is critical for phosphorylation of the ribosomal S6 protein, a protein involved in the efficient translation of critical cell-cycle regulatory proteins (Sehgal et al 1994; Wood & Bierer 1994). Rapamycin-FKBP12 complex does not inhibit $p70^{S6}$ kinase activation in a cell-free system, suggesting that rapamycin may mediate its inhibition by direct or indirect inhibition of other phosphatases (Wood & Bierer 1994). Rapamycin disrupts a Ras-dependent signal transduction pathway required for the activation of the $p70^{S6}$ kinase.

The initial activation step of peripheral T lymphocytes results in expression of all three G1 cyclins (D2, E and A) together with their appropriate cdk partners (cdk4, cdk6 and cdk2 respectively) (Firpo et al 1994). The activity of the cyclin-cdk complexes depends on a signal provided by IL-2 and other growth promoting cytokines. Rapamycin significantly decreases the kinase activity of the cyclin-dependent kinase 4-cyclin D and cyclin-dependent kinase 2-cyclin E complexes (Firpo et al 1994; Sehgal et al 1994; Wood & Bierer 1994) which normally reach their peak in the mid to late G1 phase (Sherr 1994). The activation of the cyclin-dependent kinases during cell cycle progression involves a change in their stoichiometry with the cyclin-dependent kinase inhibitors p21 and $p27^{kip1}$. Rapamycin blocks removal of the kinase inhibitor p27 from the cdk/cyclin complexes, thus preventing the activation of the cyclin-dependent kinase-cyclin complexes (Nourse et al 1994). Thus, the block in the cell cycle progression into the G1 phase is partly due to the persistance of high levels of $p27^{kip1}$ in activated T cells. IL-2 stimulation overcomes the $p27^{kip1}$ induced block by inducing both a progressive decrease in $p27^{kip1}$ protein levels and the assembly of additional G1 cyclin-cdk complexes (Firpo et al 1994; Nourse et al 1994). The degradation of $p27^{kip1}$ occurs via an ubiquitin-proteasome pathway (Pagano et al 1995). Rapamycin blocks the

downregulation of the Kip1 protein which is normally induced by IL-2 and as a result the G1 cyclin-cdk complexes remain saturated with $p27^{kip1}$ and are unable to execute critical regulating functions such as hyperphosphorylation of the retinoblastoma protein (Terada et al 1993) as well as preventing disassociation of the retinoblastoma-E2F complex which occurs following activation of cdk4/cyclin D and cdk2/cyclin E complexes. As a result, there is a decreased synthesis of cell cycle proteins, cdc2, cyclin A (Flanagan & Crabtree 1993) and TTK, a serine threonine tyrosine kinase (Schmandt et al 1994), events that are important in enabling progression through the G1 phase of the cell cycle. mTOR, the target of the FKBP12-Rapamycin complex is a critical component of the signaling pathway marking the Kip1 protein for ubiquitin-dependent proteolysis (Abraham & Wiederrecht 1996).

Rapamycin has been shown to inhibit $p70^{56}$ kinase mediated activation of CREM τ, a member of the cAMP-responsive element binding factor (CREB/ATF) family, thereby blocking cAMP-induced late gene transcription (de Groot et al 1994). Rapamycin has also been shown to selectively block IL-2 –induced transcription of proliferating cell nuclear antigen (PCNA), an obligate cofactor of DNA polymerase-δ, an important component for DNA replication (Feuerstein et al 1995). Rapamycin inhibited the binding of CREB/ATF transcription factors to CRE elements in the murine proximal PCNA promotor, suggesting that rapamycin may inhibit PCNA gene expression by preventing the interaction of CREB/ATF transcription factors with CRE elements in the proximal PCNA promotor (Feuerstein et al 1995).

Rapamycin inhibits CD28 mediated down-regulation of IκBα resulting in the inhibition of nuclear translocation of c-Rel, a CD28 response element-binding factor that causes sustained upregulation of IL-2 gene expression (Lai & Tan 1994).

3.1.2. Effect on lymphocyte proliferation

Rapamycin inhibits both calcium-dependent and -independent T cell activation pathways (Sehgal et al 1994; Wood & Bierer 1994).

In vitro studies have shown that rapamycin inhibits murine, porcine and human T lymphocyte proliferation in response to the following stimuli: mitogenic lectins, antigen, cross-linking of receptors with monoclonal antibodies (anti-CD3 and anti-CD28 antibodies), alloantigen, phorbol ester, calcium ionophore and cytokines (IL-2,

IL-4 and IL-6). The IC_{50} values range from 0.1 – 300 nM depending on the stimulator (Sehgal & Bansbach 1993; Sehgal et al 1994; Wood & Bierer 1994; Sehgal 1995, 1998). Rapamycin inhibits proliferation of activated T cells when the drug is added up to 12 hours after stimulation, whereas Cyclosporin and FK506 are ineffective when added 2 hours after activation (Dumont et al 1990b; Sigal & Dumont 1992; Sehgal & Bansbach 1993). IL-12, IL-7 and IL-15 dependent T cell proliferation is inhibited by rapamycin (Bertagnolli et al 1994; Sehgal et al 1994). Thus, unlike Cyclosporin and FK506 which block calcium-dependent cytokine transcription early in the G_1 phase, rapamycin inhibits cytokine mediated signal transduction pathways later in the G_1 phase. (Feuerstein et al 1995).

Rapamycin is able to induce T cell clonal anergy even in the presence of full T cell activation (Signal 1 and 2) by interrupting the biochemical events which would occur during the progression through the cell cycle from G1 into the S phase (Powell et al 1999).

Rapamycin has been shown to selectively promote expansion of functional human $CD4^+$ $CD25^+$ $FOXP3^+$ and deplete $CD4^+$ $CD25^-$ T effector cells (Battaglia et al 2005, 2006; Strauss et al 2007). Rapamycin induces in vitro FOXP3 expression in activated murine $CD4^+$ $FOXP3^-$ T cells (Gao et al 2007). Rapamycin, but not Cyclosporin, enables thymic generation and peripheral preservation of murine $CD4^+CD25^+FOXP3^+$ Tregs (Coenen et al 2007a). Rapamycin has also been shown to simultaneously promote TGF-β-mediated generation of murine $FOXP3^+$ Tregs and inhibit TGF-β/IL-6 mediated differentiation of CD4 Th17 cells (Kopf et al 2007). In a human MLR model, Rapamycin does not inhibit the induction of FOXP3 gene transcription (Baan et al 2005), but has been shown to inhibit the expansion of naturally occurring Tregs (Lim et al 2007). In renal transplant patients, Rapamycin did not alter the generation of $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs (Korczak-Kowalska et al 2007). In a MLR model, Rapamycin enhances the number of alloantigen-induced human $CD103^+$ $CD8^+$ T regulatory cells (Uss et al 2007).

Rapamycin also suppresses natural killer, lymphokine-activated killer and antibody-dependent cell cytotoxicity functions of human lymphocytes, but at concentrations that are 10-100 fold greater than those used to block T cell proliferation (Sehgal et al 1994).

Rapamycin is able to preserve or promote activation induced cell death of peripheral T cells (Wells et al 1999; Li et al 1999; Takahashi et al 2004; Gao et al 2007).

Rapamycin inhibits IL-2 dependent as well as independent proliferation of purified normal human B cells in response to *Staphylococcus aureus* and soluble CD40L in the mid-G1 phase of the cell cycle (Aagaard-Tillery & Jelinek 1994). It prevents *Staphylococcus aureus*-induced and IL-2 or IL-6 dependent B cell differentiation into antibody-producing cells, thereby resulting in a decrease in production of immunoglobulins M, G and A (Kim et al 1994).

3.1.3. Effect on cytokine production and response

Unlike Cyclosporin and Tacrolimus, rapamycin has limited effects on cytokine production. It does not inhibit the transcription of IL-2, IL-3 and IL-4, granulocyte-macrophage colony stimulating factor, TNF-α or interferon-γ in mitogen-activated T cells.

Rapamycin has no effect on the expression of early response genes such as c-fos, c-jun or c-myc, but inhibits transcription of bcl-2, a proto-oncogene which is induced by IL-2 and which may be critical for cell-cycle progression (Miyazaki et al 1995).

In contrast to its limited effects on cytokine production, rapamycin blocks proliferative responses to IL-1, IL-2, IL-3, IL-4, IL-6, basic fibroblast growth factor, stem cell factor, platelet-derived growth factor, insulin growth factor-1, colony-stimulating factors and IL-12 (Sehgal et al 1994; Wood & Bierer 1994; Bertagnolli et al 1994).

3.1.4. Effect on protein synthesis

Progression of mitogen-stimulated cells through the G1 phase is dependent on regulated alterations in both transcription of specific genes and the translation of certain mRNA transcripts. The initiation of translation is frequently the rate limiting step in protein synthesis (Hershey 1991; Sonenberg 1993). Eukaryotic mRNA's have a 7-methylguanylate cap at the 5′-terminus and this cap region acts as the recognition site for the eukaryotic initiation factor (eIF)-4F complex. The cap-binding protein, eIF-4E, is an important target of protein kinases involved in controlling initiation of translation.

The binding activity of eIF-4E is regulated by its interaction with PHAS-I, a heat stable inhibitor of eIF-4E function (Pause et al 1994; Lin et al 1994). Stimulation of cells with growth factors such as IL-2 results in hyperphosphorylation of PHAS-I and its release of the associated eIF-4E. Mitogenic stimuli enhance the initiation of translation by activating protein kinases which phosphorylate PHAS-1. Rapamycin blocks the increase in phosphorylation in PHAS-I and the release of active eIF-4E, induced by IL-2 or insulin (Lin et al 1995), thereby blocking eIF-4E dependent initiation of translation. Rapamycin has been shown to have a selective suppressive action on mitogen-induced protein synthesis, with rapamycin specifically interfering with the synthesis of proteins from a class of mRNAs bearing the polypyrimidine tracts at the 5′ termini (Jefferies et al 1994; Terada et al 1994). These polypyrimidine-containing mRNAs are transcripts that encode ribosomal proteins and elongation factors suggesting that rapamycin has a suppressive effect on the components of the protein synthetic machinery itself (Abraham & Wiederrecht 1996).

3.1.5. Effect on non-immune cell proliferation

Rapamycin also inhibits growth factor-mediated proliferation of non-immune cells. It blocks basic fibroblast growth factor-induced proliferation of bovine aortic and human umbilical vein endothelial cells and 3T3 fibroblasts (Akselband et al 1991). It also inhibits basic fibroblast growth factor and platelet-derived growth factor stimulated proliferation of smooth muscle cells (Gregory et al 1993; Marx et al 1995).

Rapamycin has been shown to inhibit tumour growth as a result of cell cycle arrest and the induction of apoptosis (Luan et al 2002). Rapamycin is able to downregulate anchorage-independent growth through inhibition of the eIF4E growth factor (Bjornsti et al 2004).

Rapamycin has been shown in murine models to inhibit primary and metastatic growth by antiangiogenesis. The antiangiogenic activities are linked to a decrease in production of vascular endothelial growth factor and to a markedly inhibited response of vascular endothelial cells to stimulation by VEGF (Guba et al 2002).

In a mouse model, rapamycin conditioning of renal cancer cells upregulated E-cadherin expression and altered the phenotypic expression from invasive spindle cells to non-

invasive cuboidal cells that formed cell-cell adhesions, preventing metastatic spread. Rapamycin increased $p27^{kip1}$, reduced cyclin 1, and arrested growth in the G1/S phase in this model (Luan et al 2002).

4. INTERACTIONS BETWEEN CYCLOSPORIN, FK506 AND RAPAMYCIN

In vitro combination studies have shown that Cyclosporin and rapamycin act synergistically in the inhibition of T cell and B cell proliferation (Kahan et al 1991). Equimolar concentrations of FK506 and Rapamycin inhibit Concanavalin A induced T cell proliferation in an additive manner. Rapamycin has been shown at a 50-100 M excess to block the immunosuppressive effects of FK506 on T and B cell proliferation assays (Bierer et al 1990a; Wicker et al 1990) on T cell IL-2 production, IL-2Rα expression, exocytosis, NF-AT-dependent transcription and apoptosis (Bierer et al 1990a, Staruch et al 1991) and exocytosis of human neutrophils and basophils (DePaulis et al 1991; Forrest et al 1991). FK506 at a 100-fold M excess is able to block the immunosuppressive effects of rapamycin (Dumont et al 1990a). The immunosuppressive effects of Cyclosporin on T cell responses are always enhanced by the addition of FK506 or rapamycin independent of the drug concentration (Metcalfe & Richards 1990). The reciprocal antagonism of rapamycin and FK506 reflects the competition between the two macrolides for a common intracellular binding site (Bierer et al 1990a; Dumont et al 1990a) on the immunophilin FKBP-12.

CHAPTER FIVE:

EXPERIMENTAL SECTION: MODULATION OF LYMPHOCYTE PROLIFERATION BY Ba100

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1.1. Experimental approach

2. Results

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(i) Effect of Ba100 on PHA-induced proliferation

(ii) Effect of pre-incubation with Ba100 on PHA-induced PBMC proliferation

(iii) Effect of Ba100 on PHA-induced PBMC proliferation when added 2 h after PHA

(iv) Effect of washing away Ba100 on PHA-induced PBMC proliferation

2.1.1. Lymphocyte populations and phenotype of the lymphocytes after PBMC proliferation in response to PHA in the absence or presence of Ba100

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2.3. PBMC proliferation to alloantigens in a primary mixed lymphocyte reaction (MLR)

2.3.1. Primary mixed lymphocyte reaction (MLR)

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3. Discussion

CHAPTER FIVE:

EXPERIMENTAL SECTION: MODULATION OF T LYMPHOCYTE PROLIFERATION BY Ba100

1. INTRODUCTION

Our initial studies investigating the bioactivity of the novel C-type lectin, Ba100, that we isolated from the Western Cape, South African puff adder, *Bitis Arietans*, looked at interactions with platelets (Jennings et al 1999). Although Ba100 did not aggregate washed platelets, it inhibited dose-dependently (10 - 1000 nM) aggregation of platelets in platelet-rich plasma using ADP as a stimulant. 50% inhibition was achieved at a concentration of 150 nM. Ba100 also reduced the rate of clot formation in a dose-dependent manner as assessed by measuring the α angle of the thromboelastogram (Jennings et al 1999). This inhibition of platelet aggregation and effect on clot formation was found to be due to its fibrinogenase activity that cleaves the Aα and Bβ chains of fibrinogen preventing fibrinogen crosslinking and fibrin clot formation.

As discussed in Chapter 1, C-type lectins play an important role in the immune system (Zelensky & Gready 2005) and plant lectins such as Concanavalin A (Con A) and Phytohaemagglutinin (PHA) are known stimulators of T lymphocyte proliferation. T cell activation leading to the generation and proliferation of effector T lymphocytes involves a number of inter-related steps, including: i) early signal transduction events ii) transcriptional activation of various genes iii) expression of new cell surface adhesive/costimulatory molecules iv) secretion of effector cytokines/chemokines and/or performance of cytolytic functions and v) induction of mitotic activity. Antigen-specific interactions between antigen-presenting cells (APCs) and T lymphocytes leading to full activation and consequent T lymphocyte proliferation is dependent on antigen recognition involving the TCR/CD3 complex, cellular adhesion and costimulation. CD25 (α chain of the interleukin-2 receptor) is expressed in the early phase of T cell proliferation and the clonal proliferation of activated T cells depends on the expression of this receptor (Taniguchi & Minami 1993). In addition to the early expression of CD25 on T cells, APC-T cell interactions are further enhanced through the upregulation

of bidirectional cell surface adhesive/costimulatory molecules such as LFA-1 (CD11a/CD18) interacting with CD54 (ICAM-1) (Mackay & Imhof 1993). Besides stabilizing APC-T cell interactions, these adhesive/costimulatory molecules provide important costimulatory signals for TCR-mediated activation of resting T cells (van Seventer et al 1990). TCR stimulation is insufficient to sustain full activation of T cell and additional signals are required (Steinman & Young 1991). Antigen-presenting cells are important as they deliver co-stimulatory signals to T cells which, via independent intracellular pathways, synergise with TCR stimulation leading to cytokine production and T cell activation and proliferation (Schwartz et al 1989).

Engagement of the T cell receptor and activation of co-stimulatory signals such as the CD28 co-stimulatory pathway (Ledbetter et al 1990; June et al 1994; Solomon & Bluestone 2001; Sharpe & Freeman 2002) allows gene transcription and the production of cytokines that enable T cells to move from a resting G0 to the activated G1 phase. The progression from G1 through to the S phase is regulated through a variety of receptors on the T cell surface including the IL-2 receptor. The binding of T cell growth factors such as IL-2 to the high affinity receptors, initiates signaling events required for the progression of the T cell in the G1 phase into the S phase and ultimately into mitosis. The enzymes critical for the induction of cell division include the cyclin/cyclin-dependent kinases and de novo purine and pyrimidine synthesis is essential before lymphocytes can complete cell division.

The mitogenic lectins, Concanavalin A (Con A) and Phytohaemagglutinin (PHA) induce signals through the TCR leading to increased intracellular calcium and consequent IL-2 production and IL-2 receptor expression (Vermot Desroches et al 1991; Komada et al 1996). The expression of CD25, HLA-DR, LFA-1 and ICAM-1 is upregulated on T lymphocytes proliferating in response to PHA or Con A (Vermot Desroches et al 1991; Barten et al 2001). Con A is also able to bind to the CD2 receptor and activate protein kinase C (Imboden & Stobo 1985). PHA induces signals through the crosslinking of LFA-1, which acts synergistically with the TCR/CD3 complex to induce tyrosine phosphorylation and activation of the focal adhesion kinase pathway (Tabassam et al 1999 a, b)

In view of its C-type lectin structure and its protease/fibrinogenase activity on fibrinogen, a multifunctional protein interacting with a variety of cells, we postulated

that Ba100 might also have immunomodulatory potential. The aim of this chapter was to investigate whether Ba100 was able to inhibit T lymphocyte proliferation as well as the consequences of T cell activation such as the induction of T cell activation markers and adhesive/costimulatory molecules, cytokine production and cell cycle progression. Standard T lymphocyte proliferation assays assessing both calcium-dependent and -independent T lymphocyte proliferation pathways were used to investigate the effect of Ba100 on T lymphocyte proliferation.

1.1. Experimental approach

The initial studies investigated the effect of Ba100 on T lymphocyte proliferation in response to the mitogen, PHA. As significant inhibition of proliferation was observed, further studies focused on establishing the best inhibitory concentration of Ba100 and whether this protein modulated responses early or late in T lymphocyte proliferation.

Ba100 modulation of proliferation in response to recall antigens, allo-antigens in a mixed lymphocyte reaction (MLR) as well as proliferation occurring as a result of calcium-independent T cell activation pathways (T lymphocyte proliferation in response to anti-CD28 antibody and a phorbol ester, phorbol 12-myristate 13-acetate (PMA)) was then investigated.

The effect of Ba100 on lymphocyte populations following stimulation with PHA or in a MLR as well as the expression of various receptors on responding lymphocytes was investigated using flow cytometry. Particular emphasis was placed on:

i) Cell surface activation markers

- CD25 (Interleukin-2 receptor α chain) which is expressed in the early phase after T cell activation. The clonal proliferation of activated T cells depends on the expression of this receptor (Taniguchi & Minami 1993).

- HLA-DR which is expressed on activated T lymphocytes and is involved in co-operational cellular interactions between lymphocytes and macrophages.

ii) Adhesion/stimulatory molecules

- CD2 which binds to LFA-3 (CD53) and augments lymphocyte proliferation probably via a direct effect on the TCR (Davis & van der Merwe 1996; Yashiro et al 1998).
- CD11a, a component of CD11a/CD18 (LFA-1), the counter receptor for CD54 (ICAM-1). Both are bidirectionially expressed on the surface of APCs and T lymphocytes (Mackay & Imhof 1993). LFA-1–ICAM-1 binding provides a costimulatory signal for TCR-mediated activation of resting T lymphocytes (van Seventer et al 1990). CD11a also interacts with ICAM-2 and ICAM-3 augmenting adhesion and TCR activation (Zuckerman et al 1998).
- CD49d/CD29 (VLA-4) which interacts with VCAM-1 on endothelium and fibronectin promoting lymphocyte adhesion and transendothelial migration. This receptor is upregulated during lymphocyte proliferation (Wayner et al 1989; Elices et al 1990).

iii) Costimulatory molecule CD28:

- The CD28/B7 pathway is the key positive costimulatory pathway (Salomon & Bluestone 2001; Sharpe & Freeman 2002).

The functional capacity of lymphocyte subpopulations activated in primary and secondary MLR cultures (induction of cytolytic lymphocytes and alloantigen-induced suppressor cells) in the presence of Ba100 was assessed.

Having demonstrated the ability of Ba100 to inhibit calcium-dependent T lymphocyte proliferation pathways, inhibit the generation of lymphocytes expressing activation markers and downregulate the expression of adhesive/costimulatory molecules, we then investigated the effects of Ba100 on:

i) Intracellular cytokine production during lymphocyte proliferation.

ii) Cell cycle progression

Our observation that Ba100 did cause cell cycle arrest, resulted in an investigation of the effect of Ba100 on proliferating cell nuclear antigen (PCNA) expression (an auxiliary protein necessary for DNA polymerase and cell cycle progression into the S phase).

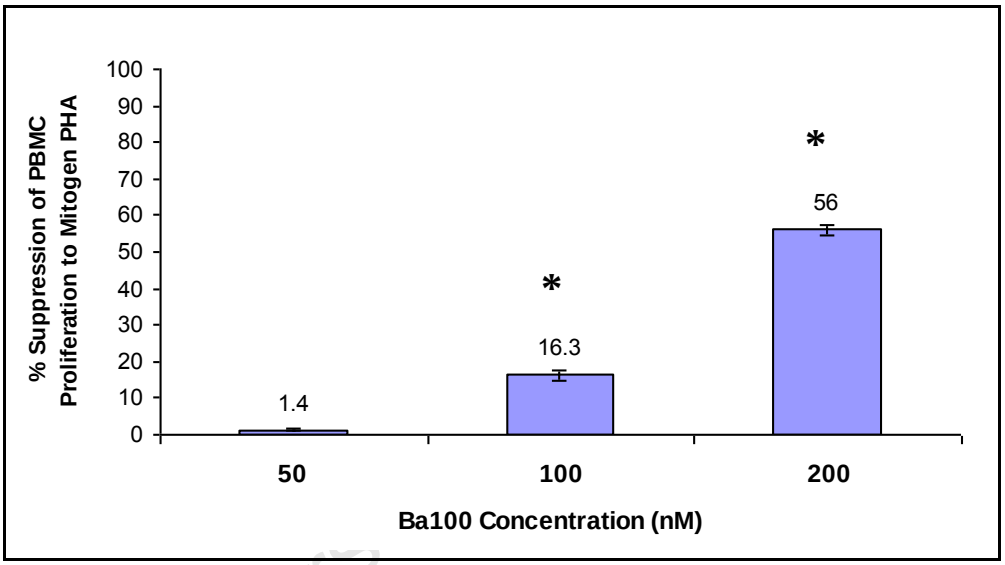
2. RESULTS

2.1. PBMC proliferation assays in response to the mitogen, PHA

i) Effect of Ba100 on PHA-induced PBMC proliferation

The effect of various concentrations of Ba100 on proliferation of PBMC in response to PHA was investigated and the data from a representative experiment is shown in Figure 1.

Figure 1. Effect of various concentrations of Ba100 on PHA-induced PBMC proliferation.



PBMC ($1x10^5$ cells/well) were incubated with PHA (0.00575 mitogenic units) or PHA + Ba100 (200, 100 and 50nM concentrations) at 37° C.

Ba100 was preincubated with PBMC for 30 minutes at 37°C prior to the addition of PHA.

At 72h, [^{3}H] thymidine was added, incorporation measured 18h later and results are expressed as mean DPM of 6 replicates ± SD.

Background [^{3}H] thymidine incorporation into PBMC in absence of PHA or presence of Ba100 were 210 ± 10 DPM and 280 ± 35 DPM respectively. [^{3}H] thymidine incorporation into PHA-stimulated PBMC was 169 660 ± 2840 DPM.

Results of a representative dose response to Ba100 are shown and are expressed as % suppression of PBMC proliferation to PHA ± SD based on the PHA-stimulated control. * $p < 0.0001$, comparing % suppression at each successive concentration.

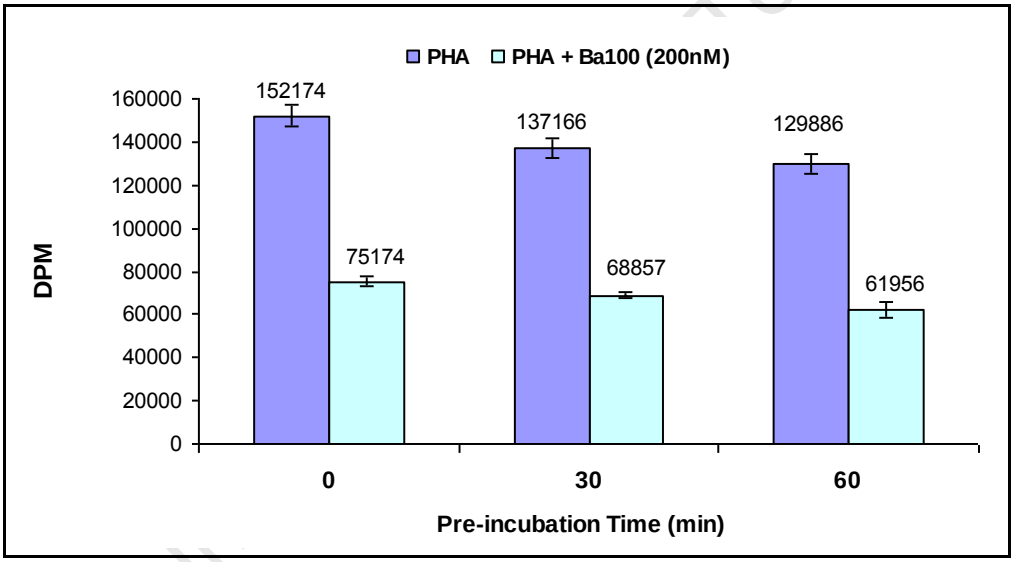
In the absence of PHA, Ba100 did not alter background [^{3}H] thymidine incorporation. When Ba100 was preincubated with PBMC for 30 min prior to the addition of PHA, a dose dependent inhibition of the proliferation of the PBMC was observed (Figure 1). 2 further dose response experiments confirmed a simple linear relationship between the concentration of Ba100 and % suppression of PBMC proliferation in response to PHA ($p < 0.0001$) and the IC50 was ± 169nM. A further set of 10 experiments was done to

confirm the effect of a 30 min preincubation period of Ba100 (200nM) on PHA-induced PBMC proliferation. For each experiment, inhibition of proliferation was observed and the range of suppression of proliferation for these 10 experiments was 45 – 67% (mean 56.6%, median 58% suppression). Exclusion of trypan blue by cells harvested at 72 h indicated that the cells were not being killed by Ba100 and thus the inhibition of proliferation was not due to cytotoxicity.

ii) Effect of pre-incubation with Ba100 on PHA-induced PBMC proliferation

We investigated whether pre-incubation with Ba100 was required for the inhibitory effect on PHA-induced PBMC proliferation.

Figure 2. Effect of pre-incubation with Ba100 on PHA-induced PBMC proliferation.



PBMC (1x10^5 cells/well) were incubated with PHA (0.00575 mitogenic units) or PHA + Ba100 (200nM) at 37°C.
Ba100 was either pre-incubated for 30 min or 60 min with PBMC at 37°C prior to the addition of PHA or added to the PBMC when the PHA was added.
At 72 h, [^{3}H] thymidine was added and incorporation measured 18h later.
Background [^{3}H] thymidine incorporation for cells only or cells + Ba100 (200 nM) never averaged above 334 DPM.
Results of a representative experiment are shown and are expressed as mean DPM of 6 replicates ± SD.

We found that pre-incubation of PBMC with Ba100 was not required for its anti-proliferative effect (Figure 2). The percentage suppression of PHA-induced PBMC proliferation with a 30 min and 60 min preincubation period (49.8% ± 1.3 (SD) and 52.3% ± 1.12 (SD) suppression respectively) was the same as that when Ba100 was added at the time of addition of PHA (50.6% ± 1.2 (SD) suppression). This was

confirmed in 2 further experiments (regression coefficient 0.005, SE 0.016). For standardization purposes, a 30 minute pre-incubation period was used in all the experiments, unless otherwise stated.

iii) Effect of Ba100 on PHA-induced PBMC proliferation when added 2h after PHA

The ability of Ba100 to modulate PHA-induced PBMC proliferation after the stimulus for proliferation had been added, was investigated. To assess whether Ba100 acted early or late in T cell signal transduction, Ba100 was added 2 hours after the addition of the mitogenic stimulus, PHA.

Table 1. Effect of Ba100 on PHA-induced PBMC proliferation when added 2h after PHA.

Reaction		[^{3}H] Thymidine incorporation (DPM)	Suppression of PBMC proliferation to PHA
Mitogen	**Ba100 (200 nM)**		
PHA	-	354 418 ± 16317	-
PHA	30 min pre-incubation	184 297 ± 2493	48% ± 0.71
PHA	Added 2h after PHA	349 534 ± 3660	0%

PBMC ($1x10^5$ cells/well) were incubated with PHA (0.00575 mitogenic units) or PHA + Ba100 (200 nM) at 37°C.

Ba100 was preincubated with PBMC for 30 min at 37°C prior to the addition of PHA or Ba100 was added 2 h after PHA.

At 72 h, [^{3}H] thymidine was added and incorporation measured 18h later.

Background [^{3}H] thymidine incorporation into PBMC in the absence of PHA or presence of Ba100 were 274 ± 20 DPM and 344 ± 55 DPM respectively.

Results of a representative experiment are shown and are expressed as mean DPM of 6 replicates ± SD or % suppression of PBMC proliferation to PHA ± SD.

Ba100 was unable to inhibit PHA-induced PBMC proliferation if added 2h after PHA (Table 1), suggesting that Ba100 exerted its inhibitory effect early on in T cell activation. This was reproducible in two further experiments.

iv) Effect of washing away Ba100 on PHA-induced PBMC proliferation

The effect of preincubating Ba100 with PBMC, then washing it away before the addition of PHA to initiate proliferation, was investigated.

Table 2. Effect of washing away Ba100 on PHA-induced PBMC proliferation.

Reaction		[^{3}H] Thymidine incorporation (DPM)	Suppression of PBMC proliferation to PHA
Mitogen	**Ba100 (200 nM)**		
PHA	-	142 096 ± 7754	-
PHA	30 min pre-incubation	70 988 ± 1473	50% ±1.41
PHA	30 min pre-incubation, then wash	153 309 ± 8420	0%

PBMC (1x10^5 cells/well) were incubated with PHA (0.00575 mitogenic units) or PHA + Ba100 (200 nM) at 37°C.

Ba100 was pre-incubated with PBMC for 30 min at 37°C prior to the addition of PHA. For one reaction Ba100 was washed away after the 30 min pre-incubation period.

At 72 h, [^{3}H] thymidine was added and incorporation measured 18h later.

Background [^{3}H] thymidine incorporation into PBMC in the absence of PHA or presence of Ba100 were 377 ± 30 DPM and 385 ± 55 DPM respectively.

Results of a representative experiment are shown and are expressed as mean DPM of 6 replicates ± SD or % suppression of PBMC proliferation to PHA ± SD.

Removing Ba100 from the reaction prior to the addition of PHA resulted in a complete loss of the inhibitory effect of Ba100 on PHA-induced PBMC proliferation (Table 2). Ba100 needs to be present during the proliferation period for its inhibitory effect to be observed. This was reproducible in two further experiments.

2.1.1. Lymphocyte populations and phenotype of the lymphocytes after PBMC proliferation in response to PHA in the absence or presence of Ba100

Flow cytometry was used to analyse the phenotype of the lymphocyte population after proliferation of PBMC in response to PHA in the absence or presence of Ba100 (200 nM). Both the % of cells in the lymphocyte gate (set on lymphocyte forward and side scatter properties) expressing a particular receptor and the MFI (mean fluorescent intensity) of the antibody binding was determined. Lymphocytes in the lymphocyte gate were analysed for expression of activation markers HLA-DR and the interleukin 2 receptor (CD25); adhesion receptors CD2, CD11a, CD49d and CD29; co-stimulatory molecules CD28; CTL markers in the form of S6F1 and NK cell markers in the form of CD56. Anti-CD19 was used to detect B cells and anti-CD3 to detect T cells. Anti-CD4 and anti-CD8 was used to determine T cell subsets.

Table 3. Lymphocyte populations and phenotype after proliferation of PBMC in response to PHA in the absence or presence of Ba100 (200 nM).

Receptors	PHA-stimulated cells -Ba100		PHA-stimulated cells + Ba100 (200 nM)		% Change + Ba100 (200 nM)	
	% Positive cells	MFI	% Positive cells	MFI	% Positive cells	MFI
CD3	78.5	8.3	74.9	7.9	No change	No change
CD4	47.1	18.3	49.8	19.2	No change	No change
CD8	33.4	17.7	28.1	14.1	15.9% ↓	20.3% ↓
CD56	8.8	4.6	8.3	4.5	No change	No change
S6F1	17.8	30.1	9.9	11.6	44.4% ↓	61.5% ↓
CD19	15.2	4.1	14.3	3.6	No change	No change
HLA-DR	58.4	22.5	19.5	13.5	66.6% ↓	40.0% ↓
CD25	87.2	21.5	62.5	6.2	28.3% ↓	71.2% ↓
CD2	87.2	13.1	86.2	12.1	No change	No change
CD11a	52.8	11.8	31.5	7.4	40.3% ↓	37.3% ↓
CD49d	38.5	4.4	18.3	2.9	52.5% ↓	34.1% ↓
CD29	56.6	6.3	22.4	4.3	60.4% ↓	31.7% ↓
CD28	29.5	7.0	3.0	4.4	89.8% ↓	37.1% ↓
CD4 : CD8	1.4		1.8		28.6% ↑	

PBMC ($1x10^5$ cells/well) were incubated with PHA (0.00575 mitogenic units) or PHA + Ba100 (200 nM) at 37°C.

Ba100 was pre-incubated for 30 min with PBMC at 37°C prior to the addition of PHA.

At 72 h, the cells were harvested, then labeled with antibodies directed to the indicated lymphocyte receptors and single colour analysis of antibody binding was done by flow cytometry on gated lymphocytes.

Results of a representative experiment are shown and are expressed as % of cells in the lymphocyte gate expressing the receptor and the MFI (mean fluorescent intensity or number of receptors per cell) of the antibody binding. The change in these parameters in the presence of Ba100 is expressed as a % of that in the absence of Ba100. ↓ indicates a decrease from that in the absence of Ba100 and ↑ indicates an increase from that in the absence of Ba100.

A change in the % of cells expressing a specific receptor and a change in MFI in the presence of Ba100 was considered a real change if this was more than a 10% and 20% change respectively. Only real changes decided by these two criteria are shown in the last 2 columns.

From the results of a representative experiment given in Table 3, it appears that PBMC proliferation in response to PHA in the presence of Ba100 resulted in impaired generation of lymphocytes that expressed:

i) activation markers HLA-DR and CD25.

ii) adhesion molecules CD11a, CD49d and CD29.

iii) the co-stimulatory molecule CD28.

iv) the CTL marker S6F1.

In the presence of Ba100, for those lymphocytes that did express these receptors, the number of receptors expressed per cell was reduced. There was no change in the percentage of B cells in the lymphocyte population. An increase in the CD4:CD8 ratio was seen.

This impaired generation of lymphocytes that expressed these receptors was confirmed in a further 5 experiments after proliferation of PBMC in response to PHA in the presence of Ba100 (200 nM). The range of suppression and median suppression for both percentage of cells expressing a receptor and the number of receptors expressed per cell are tabulated in Table 4. From the data, it is clear that these results are consistent with those shown in Table 3, except that in this set of experiments, no change in the CD4:CD8 ratio was detected.

Table 4. Change in lymphocyte populations and phenotype after proliferation of PBMC in response to PHA in the presence of Ba100 (200 nM).

Receptors	PHA-stimulated PBMC + Ba100 (200 nM)			
	% Positive cells		MFI	
	Range of suppression	Median	Range of suppression	Median
HLA-DR	51.2 – 73.3%	64.6%	38.0 – 41.0%	38.0%
CD25	31.0 – 44.0%	32.5%	65.2 – 80.6%	65.2%
CD2	No change		No change	
CD11a	32.0 – 56.7%	42.6%	41.2 – 45.3%	41.7%
CD49d	32.7 – 50.5%	46.4%	36.5 – 42.8%	39.2%
CD29	52.3 – 73.3%	60.8%	37.4 – 40.1%	38.2%
CD28	70.2 – 85.8%	77.9%	33.7 – 41.5%	37.2%
S6F1	46.7 – 68.5%	50.3%	34.5 – 62.1%	48.0%
CD56	No change		No change	
CD19	No change		No change	
CD4 : CD8 ratio	No change		No change	

PBMC ($1x10^5$ cells/well) were incubated with PHA (0.00575 mitogenic units) or PHA + Ba100 (200 nM) at 37°C.

Ba100 was pre-incubated for 30 min with PBMC at 37°C prior to the addition of PHA.

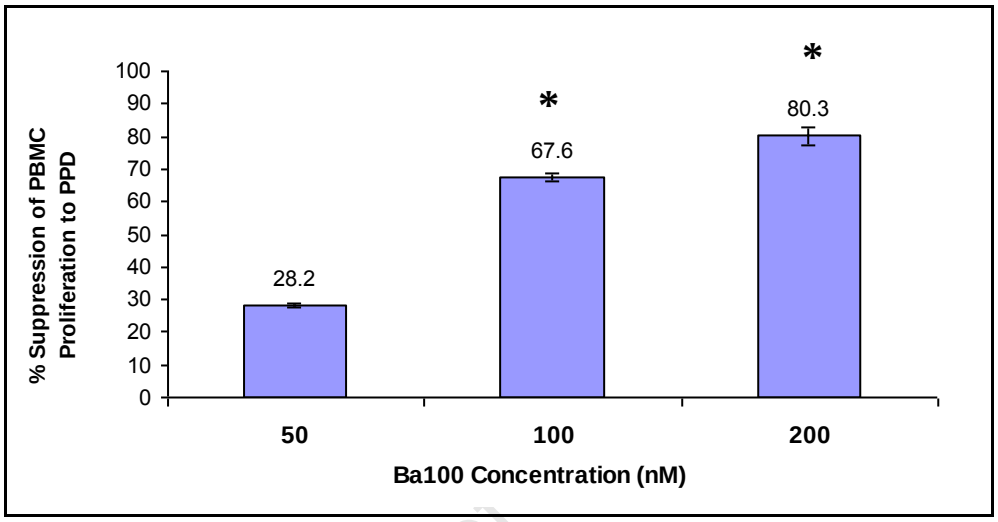
At 72 h, the cells were harvested,then labeled with antibodies directed to the indicated lymphocyte receptors and single colour analysis of antibody binding was done by flow cytometry on gated lymphocytes.

The range of suppression and the median suppression for both the % of cells in the lymphocyte gate expressing the receptor and the MFI (mean fluorescent intensity) of the antibody binding are shown.

2.2. PBMC proliferation assays in response to various antigens

The effect of Ba100 on proliferation of PBMC in response to various antigens was investigated and the data from representative experiments are shown in Figure 3a - c.

Figure 3a. Effect of various concentrations of Ba100 on PPD–induced PBMC proliferation.



PBMC ($1x10^5$ cells/well) were incubated with the antigen PPD without or with Ba100 (200, 100, 50 nM) at 37°C.

Ba100 was preincubated with PBMC for 30 min at 37°C prior to the addition of PPD.

On day 7, [^{3}H] thymidine was added, incorporation measured 18 hr later and results are expressed as mean DPM of 6 replicates ± SD.

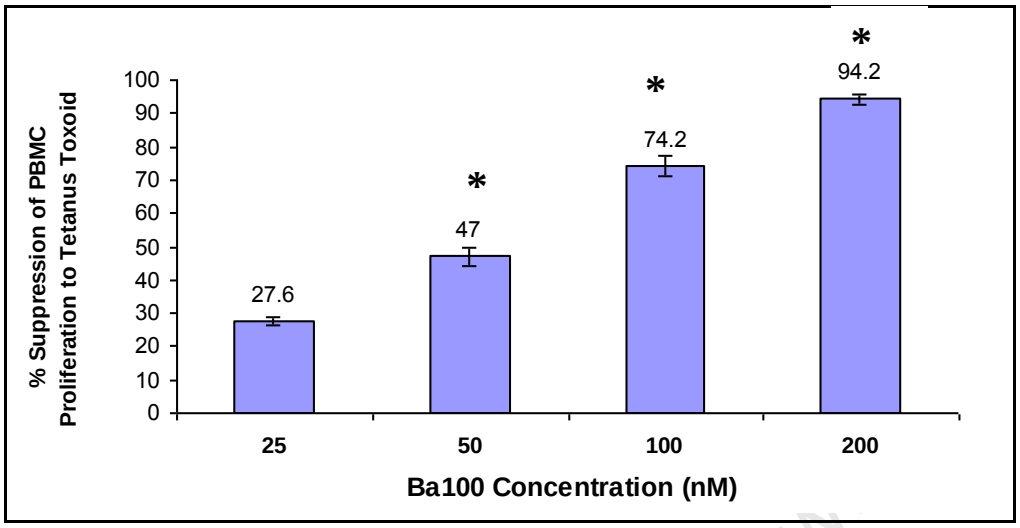
Background [^{3}H] thymidine incorporation into PBMC in the absence of PPD was 380 ± 32 DPM and in the presence of Ba100 was 376 ± 18 DPM.

[^{3}H] thymidine incorporation into PBMC in the presence of PPD was 445 432 ± 24 400 DPM.

Results of a representative dose response to Ba100 are shown and are expressed as % suppression of PBMC proliferation to PPD ± SD based on the PPD-stimulated control. * $p < 0.0005$, comparing % suppression at each successive concentration.

Ba100 dose dependently (50 – 200 nM) suppressed PBMC proliferation in response to the antigen PPD (Figure 3a). 2 further dose response experiments confirmed that the relationship between the concentration of Ba100 and % suppression of PBMC proliferation in response to PPD was linear with logarithmic transformation ($p < 0.0001$) and the IC50 was ± 83nM.

Figure 3b. Effect of various concentrations of Ba100 on Tetanus toxoid–induced PBMC proliferation.



PBMC ($1x10^5$ cells/well) were incubated with the antigen Tetanus toxoid without or with Ba100 (200, 100, 50, 25 nM) at 37°C.

Ba100 was preincubated with PBMC for 30 min at 37°C prior to the addition of Tetanus toxoid.

On day 7, [^{3}H] thymidine was added, incorporation measured 18 hr later and results are expressed as mean DPM of 6 replicates ± SD.

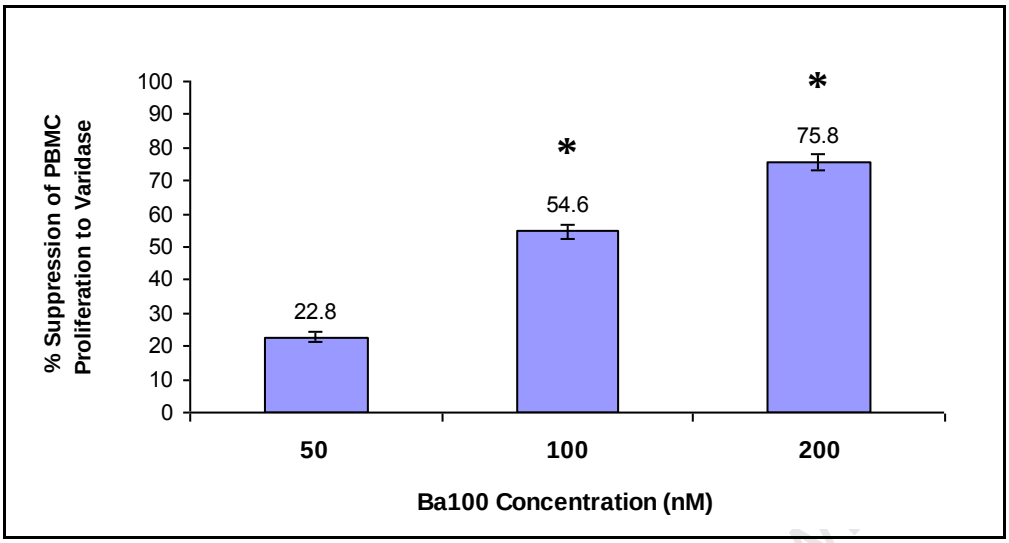
Background [^{3}H] thymidine incorporation into PBMC in the absence of Tetanus toxoid was 406 ± 52 DPM and in the presence of Ba100 was 398 ± 36 DPM.

[^{3}H] thymidine incorporation into PBMC in the presence of Tetanus toxoid was 20 397 ± 1713 DPM.

Results of a representative dose response to Ba100 are shown and are expressed as % suppression of PBMC proliferation to Tetanus toxoid ± SD based on the Tetanus toxoid-stimulated control. * $p < 0.0005$, comparing % suppression at each successive concentration.

Ba100 dose dependently (25 – 200 nM) suppressed PBMC proliferation in response to the antigen Tetanus toxoid (Figure 3b). 2 further dose response experiments confirmed that the relationship between the concentration of Ba100 and % suppression of PBMC proliferation in response to Tetanus toxoid was linear with logarithmic transformation ($p < 0.0001$) and the IC50 was ± 53nM.

Figure 3c. Effect of various concentrations of Ba100 on Varidase–induced PBMC proliferation.



PBMC ($1x10^5$ cells/well) were incubated with the antigen Varidase without or with Ba100 (200, 100, 50 nM) at 37°C. Ba100 was preincubated with PBMC for 30 min at 37°C prior to the addition of Varidase.

On day 7, [^{3}H] thymidine was added, incorporation measured 18 hr later and results are expressed as mean DPM of 6 replicates ± SD.

Background [^{3}H] thymidine incorporation into PBMC in the absence of Varidase was 390 ± 44 DPM and in the presence of Ba100 was 364 ± 32 DPM.

[^{3}H] thymidine incorporation into PBMC in the presence of Varidase was 349 584 ± 23 688 DPM.

Results of a representative dose response to Ba100 are shown and are expressed as % suppression of PBMC proliferation to Varidase ± SD based on the Varidase-stimulated control. * $p < 0.0005$, comparing % suppression at each successive concentration.

Ba100 dose dependently (50 – 200 nM) suppressed PBMC proliferation in response to the antigen Varidase (Figure 3c). 2 further dose response experiments confirmed that the relationship between the concentration of Ba100 and % suppression of PBMC proliferation in response to Varidase was linear with logarithmic transformation ($p < 0.0001$) and the IC50 was ± 95nM.

A further set of 3 experiments with each antigen was done which confirmed the inhibitory effect of a 30 min preincubation period of Ba100 (200nM) on antigen-stimulated proliferation. The range of suppression of:

i) PPD-induced proliferation was 75 – 80% (mean 77.6%, median 78% suppression).

ii) Tetanus toxoid-induced proliferation was 89 – 94% (mean 91.7%, median 92% suppression).

iii) Varidase-induced proliferation was 70 – 76% (mean 73.3%, median 74% suppression).

2.3. PBMC proliferation to alloantigens in a primary mixed lymphocyte reaction (MLR)

The immunosuppressive properties of Ba100 on T lymphocyte responses to mitogen and antigens was encouraging and suggested to us that Ba100 may be able to inhibit proliferation to alloantigens in a mixed lymphocyte reaction, which is an *in vitro* transplant model.

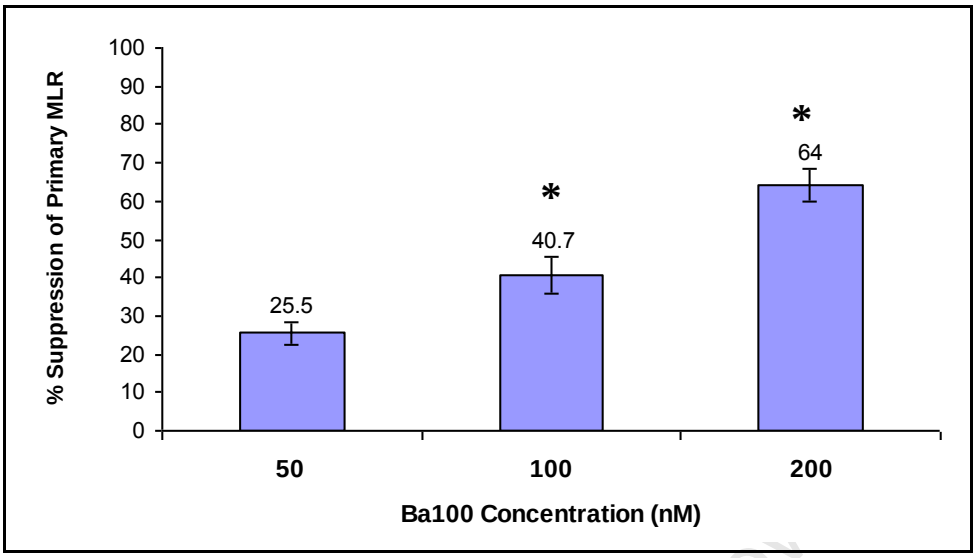
2.3.1. Primary mixed lymphocyte reaction (MLR)

The MLR provides a system for analyzing the interactions between subpopulations of antigen-presenting macrophages, allo-antigen specific CD4 lymphocytes, cytotoxic T lymphocytes and suppressor/regulatory T cells. CD4 lymphocytes constitute a major population of the responding cells in the MLR and their activity is essential for inducing other lymphocyte subsets to differentiate into effectors of cytotoxicity.

We thus looked at the potential role of Ba100 in modulating the functional activity of effector cells generated in a primary MLR. The first experiment investigated the effect of various concentrations of Ba100 on lymphocyte proliferation in a 6 day primary mixed lymphocyte reaction.

Ba100 dose dependently (50 – 200 nM) inhibited the primary mixed lymphocyte reaction (Figure 4). 2 further dose response experiments confirmed that there was a simple linear relationship between the concentration of Ba100 and % suppression of the MLR ($p < 0.0001$) and the IC50 was ± 149nM. A further set of 10 experiments with different donors was done, which confirmed the consistent inhibitory effect of a 30 min pre-incubation period of Ba100 (200nM) on the MLR. For the 10 experiments, the suppression ranged between 45% - 66% (mean 54.6%, median 54% suppression).

Figure 4. Effect of various concentrations of Ba100 on a 6 day primary mixed lymphocyte reaction.



$1x10^5$ responder cells (A) were incubated with 1 x 10^5 irradiated (3000R) stimulator cells (Bx) per well at 37°C in the absence or presence of Ba100 at varying concentrations (200, 100 and 50nM).

Responder cells were pre-incubated with Ba100 for 30 min at 37°C prior to the addition of stimulator cells.

On day 6, [^{3}H] thymidine was added, incorporation measured 18 h later and results are expressed as mean DPM of 6 replicates ± SD.

[^{3}H] thymidine incorporation for A+Bx was 74 010 ± 1401 DPM.

Results of a representative dose response to Ba100 are shown and are expressed as % suppression of proliferation ± SD based on the MLR (A+Bx) control. * $p < 0.0001$, comparing % suppression at each successive concentration.

2.3.2. Lymphocyte populations and phenotype of the lymphocytes generated in a primary MLR in the absence or presence of Ba100

Flow cytometry was used to determine the phenotype of the effector cells generated in the primary MLR in the absence or presence of Ba100 (200 nM). Both the % of cells in the lymphocyte gate (set on lymphocyte forward and side scatter properties) expressing a particular receptor and the MFI of the antibody binding was determined. Lymphocytes in the lymphocyte gate were analysed for expression of activation markers HLA-DR and the interleukin 2 receptor (CD25) and NK cell markers in the form of CD16. Anti-CD4 and anti-CD8 was used to determine T cell subsets.

Table 5. Lymphocyte populations and phenotype of effector cells generated in a 6 day primary MLR in the absence or presence of Ba100 (200 nM).

Receptor	% Positive cells		MFI		% Change (+ Ba100)	
	-Ba100	+Ba100 (200 nM)	-Ba100	+Ba100 (200 nM)	% Positive cells	MFI
Experiment 1 (A + Bx)						
CD4	42.1	61.7	5.3	6.9	46.6% ↑	30.2% ↑
CD8	18.9	17.2	17.8	7.3	No change	58.9% ↓
CD16	7.3	2.8	4.5	1.8	61.6% ↓	60.0% ↓
HLA-DR	37.2	4.7	26.6	14.6	87.4% ↓	45.1% ↓
CD25	29.4	4.3	12.2	7.2	85.4% ↓	40.9% ↓
CD4 : CD8	2.2	3.6			63.6% ↑	
Total cell yield	$4.0x10^6$/ml	$2.3x10^6$/ml				
% Suppression of proliferation		42.5%				
Experiment 2 (A + Cx)						
CD4	41.7	46.3	12.5	13.7	11.0% ↑	No change
CD8	16.5	10.4	25.8	22.9	36.9% ↓	No change
CD16	16.3	2.4	6.0	4.7	85.3% ↓	21.7% ↓
HLA-DR	44.6	10.8	31.2	18	75.8% ↓	42.3% ↓
CD25	36.8	13.2	15.1	8.1	64.1% ↓	46.4% ↓
CD4 : CD8	2.5	4.5			80.0% ↑	
Total cell yield	$4.8x10^6$/ml	$2.0x10^6$/ml				
% Suppression of proliferation		58.3%				
Experiment 3 (C + Bx)						
CD4	41.2	48.5	14.3	14.6	17.7% ↑	No change
CD8	27.5	12	45.3	15.2	56.4% ↓	66.4% ↓
CD16	21.6	2.6	11.4	8.9	87.9% ↓	21.9% ↓
HLA-DR	72.4	18.2	92.3	33.9	74.9% ↓	63.3% ↓
CD25	37.5	6.4	59.1	6.4	82.9%↓	89.2% ↓
CD4 : CD8	1.5	4.0			167% ↑	
Total cell yield	$4.0x10^6$/ml	$2.2x10^6$/ml				
% Suppression of proliferation		45%				
Experiment 4 (C + Ax)						
CD4	45.9	46	15.8	15.6	No change	No change
CD8	25.5	8.2	43.6	12.4	67.8% ↓	71.6% ↓
CD16	23.1	2.7	10.7	8.1	88.3% ↓	24.3% ↓
HLA-DR	74.2	11.3	82.6	41.7	84.8% ↓	49.5% ↓
CD25	34.2	5.2	39.4	6.0	84.8% ↓	84.8% ↓
CD4 : CD8	1.8	5.6			211% ↑	
Total cell yield	$6.0x10^6$/ml	$2.0x10^6$/ml				
% Suppression of proliferation		66.7%				

Effector cells were generated in 6 day bulk primary MLRs in the absence or presence of Ba100 (200 nM). PBMC were used from 3 different donors (A, B, C) in 4 experiments and were used as responder cells (A or C) or irradiated (3000 R) stimulator cells (Ax, Bx, Cx) as indicated. On day 6, the cells were harvested and the total cell yield determined by counting the number of cells x 10^6/ml proliferating in the MLR in the absence or presence of

Ba100 (200 nM). Aliquots of cells were then labeled with antibodies directed to the indicated lymphocyte receptors and single colour analysis of antibody binding was done by flow cytometry on gated lymphocytes.

Data from the 4 experiments are shown and are the % of cells in the lymphocyte gate expressing the receptor and the MFI (mean fluorescent intensity) of the antibody binding. The change in these parameters in the presence of Ba100 is expressed as a % of that in the absence of Ba100. ↓ indicates a decrease from that in the absence of Ba100 and ↑ indicates an increase from that in the absence of Ba100.

A change in the % of cells expressing a specific receptor and a change in MFI in the presence of Ba100 was considered a real change if this was more than a 10% and 20% change respectively. Only real changes decided by these two criteria are shown in the last 2 columns.

In summary, all 4 primary MLR (Table 5) confirmed that Ba100:

i) Suppressed the mixed lymphocyte reaction as the total cell yield on day 6 was decreased (Range of inhibition of total cell yield 42.5 - 66.7%, median 51.7%).

ii) Impaired the generation of lymphocytes expressing the activation markers: HLA-DR (range of suppression 74.9 – 87.4%, median 80.4%); CD25 (range of suppression 64.1 – 85.4%, median 83.9%);
Decreased the MFI of HLA-DR expression (range of suppression 42.3 – 63.3%, median 47.3%); and decreased the MFI of CD25 expression (range of suppression 40.9 – 89.2%, median 65.6%) per cell.

iii) Impaired the generation of NK cells as determined by the number of cells expressing CD16 (range of suppression 61.6 – 88.3%, median 86.6%) and decreased the MFI of CD16 expression (range of suppression 21.7 – 60%, median 23.1%) per cell.

iv) Increased the CD4:CD8 ratio (range of increase 63.6 - 211%, median 123.5%).

A further 4 primary MLR in the presence of Ba100 (200 nM) confirmed the impaired generation of lymphocytes that expressed the activation markers and the increase in the CD4:CD8 ratio and looked at the effect of Ba100 on the adhesion molecule CD2 and NK cells expressing CD56. The range of suppression and median suppression for the percentage of cells expressing a receptor are tabulated in Table 6.

Table 6. Change in lymphocyte populations and phenotype of effector cells generated in a 6 day primary MLR in the absence or presence of Ba100 (200 nM).

Receptors	Primary MLR + Ba100 (200 nM)	
	% Positive cells	
	Range of suppression	Median
HLA-DR	56.2 – 86.7%	67.2%
CD25	45.4 – 84.2%	72.4%
CD56	45.2 – 80.7%	74.3%
CD2	No change	
CD4 : CD8	50.6 – 100% increase	52.4%
% Suppression of proliferation	46.9 – 65.2%	55.3%

Effector cells were generated in 6 day bulk primary MLRs in the absence or presence of Ba100 (200 nM).

PBMC were used from 3 different donors (A, B, C) in 4 experiments and were used as responding or irradiated stimulator cells. On day 6, the cells were harvested and the total cell yield determined by counting the number of cells x 10^6/ml proliferating in the MLR in the absence or presence of Ba100 (200 nM). Aliquots of cells were then labeled with antibodies directed to the indicated lymphocyte receptors and single colour analysis of antibody binding was done by flow cytometry on gated lymphocytes.

The range of suppression and median suppression of the % of cells in the lymphocyte gate expressing the indicated receptor for 4 experiments are shown.

2.4. Functional analysis of lymphocytes in the primary MLR

Lymphocytes from a primary MLR cultured in the presence of Ba100 were tested for suppressor cell and CTL activity on day 6 of the culture. Cells harvested from the primary MLR were incubated for 30 min, followed by 2 washes in fresh complete media to remove Ba100 before assessment of CTL and suppressor cell activity.

2.4.1. Suppressor cell activity of the primary MLR generated effectors on a fresh MLR

The primed effector cells from the primary MLR were tested for their ability to suppress the proliferative response of the fresh responding cells in a new MLR. We looked at both specific and non-specific suppression by primed effector cells generated in the presence of Ba100.

Table 7. Effect of Ba100 on the suppressor cell assays.

Response being tested in the fresh MLR	Primed cells from a bulk primary MLR added to a fresh MLR	Fresh MLR components	[^{3}H] Thymidine incorporation (DPM)	% Suppression of proliferation of fresh MLR
1. Autologous	(A + Bx)	A + Bx	76373 ± 1359	-
Autologous	(A + Bx+ 200nM Ba100)	A + Bx	46282 ± 943	39.4% ± 1.41
2. Heterologous	(A + Bx)	C + Ex	105986 ± 1445	-
Heterologous	(A + Bx+ 200nM Ba100)	C + Ex	78854 ± 1142	25.6% ± 2.1
3. Same stimulator, different responder	(A + Bx)	C + Bx	96278 ± 1243	-
Same stimulator, different responder	(A + Bx+ 200nM Ba100)	C + Bx	63832 ± 1049	33.7% ± 1.41
4. Same responder, different stimulator	(A + Bx)	A + Cx	95755 ± 1523	-
Same responder, different stimulator	(A + Bx+ 200nM Ba100)	A + Cx	67603 ± 1012	29.4% ± 2.8

A bulk primary MLR was set up by incubating responder cells, A ($1x10^6$ cells/ml) with irradiated (3000 R) stimulator cells, Bx ($1x10^6$ cells/ml) in complete media in the absence or presence of Ba100 (200 nM) at 37°C. After 6 days incubation, the cells were harvested, incubated for 30 min, washed x 2 to remove Ba100 and used as the primed cells in the suppressor cell assay.

Fresh MLR reactions contained the component cells A+Bx, C+Ex, C+Bx, A+Cx: $1x10^5$ responder cells (A or C) were incubated with $1x10^5$ irradiated (3000 R) stimulator cells (Bx, Cx or Ex) per well in complete media at 37°C.

These fresh MLR (A+Bx, C+Ex, C+Bx and A+Cx) were cultured with the primed cells in the following combinations.
1. Primed cells autologous to responders and stimulators. 2. Primed cells, responders and stimulators completely heterologous. 3. Same stimulator, different responder. 4. Same responder, different stimulator.

On day 6, [^{3}H] thymidine was added and incorporation measured 18 h later.

Results of a representative experiment are shown and expressed as mean DPM of 6 replicates ± SD or % suppression of proliferation ± SD.

Effector cells generated in a primary MLR in the presence of Ba100 (200 nM) exerted a suppressive effect on the proliferation of a fresh MLR. This suppressor activity was not antigen specific (Table 7). This was reproducible in two further experiments.

2.4.2. Cell mediated lympholytic (cytotoxic) activity of the MLR generated effectors in a ^{51}Cr release assay

Effectors from primary MLR cultures (A + Bx or C + Bx) generated in the presence of Ba100 (200nM) were tested for their ability to kill PHA blasts (prepared from donor B) that were used as targets in a standard ^{51}Cr release assay and were compared to effectors from a MLR generated in the absence of Ba100. The generation of effectors in the MLR with cytolytic activity was reduced when Ba100 was included in the reaction. Two experiments were performed (Table 8) and in both the cytolytic activity of effector cells generated in a primary MLR in the presence of Ba100 was markedly reduced.

Table 8. Cell mediated lympholytic (cytotoxic) activity of the MLR generated effectors.

Effector: Target ratio	Experiment 1		Experiment 2	
	% Specific ^{51}Cr release		% Specific ^{51}Cr release	
	Effectors from MLR A + Bx - Ba100	Effectors from MLR A + Bx +Ba100 (200 nM)	Effectors from MLR C + Bx - Ba100	Effectors from MLR C + Bx +Ba100 200 nM)
40 : 1	47 ± 3	22 ± 3	38 ± 2	17 ± 3
20 : 1	34 ± 5	12 ± 2	33 ± 4	10 ± 3
10 : 1	19 ± 5	5 ± 1	16 ± 4	4 ± 1

Cells from a primary bulk MLR cultured in the absence or presence of Ba100 (200 nM) were harvested on day 6 and then used at the indicated effector : target ratio in a standard 4h ^{51}Cr release assay. 3-day-old PHA blast cells (prepared from donor B) and labelled with ^{51}Cr served as targets.

The data is expressed as % specific ^{51}Cr release ± SD of triplicate reactions. Activity in control reactions using targets prepared from A or C was never > 5% specific ^{51}Cr release.

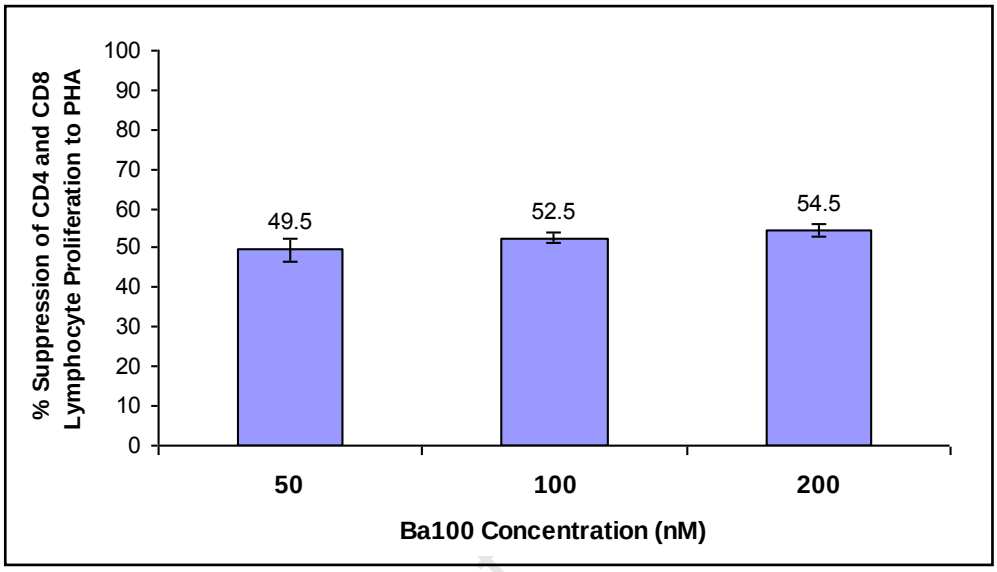
2.5. CD4 lymphocyte proliferation assays

Considering the critical role of CD4 lymphocytes in the alloimmune response, we have looked at the effect of Ba100 on the proliferation of isolated CD4 lymphocytes. We also investigated whether Ba100 needed to be processed by antigen-presenting phagocytes in order to exert its inhibitory effect on lymphocyte proliferation. CD4 and CD8 lymphocytes were isolated from PBMC using Dynal beads.

2.5.1. Effect of Ba100 on isolated CD4 and CD8 lymphocyte proliferation in response to PHA

We looked at the ability of Ba100 to inhibit CD4 and CD8 lymphocyte proliferation in response to PHA in the absence of antigen-presenting phagocytes.

Figure 5. Effect of Ba100 on isolated CD4 and CD8 lymphocyte proliferation (60:40% CD4:CD8 population ratio) in response to PHA.



CD4 and CD8 lymphocytes were isolated using Dynal beads, then reconstituted in a 60 : 40% ratio (CD4 : CD8). A total of $1x10^5$ CD4 + CD8 cells were cultured per well with PHA (0.00575 mitogenic units) or PHA + Ba100 (200, 100 and 50 nM concentrations) at 37°C.

Ba100 was pre-incubated with CD4 and CD8 lymphocytes for 30 min at 37°C prior to the addition of PHA.

At 72 h, [^{3}H] thymidine was added, incorporation measured 18 h later and results are expressed as mean DPM of 6 replicates ± SD.

Background counts in the absence of PHA or Ba100 were 326 ± 24 DPM.

[^{3}H] thymidine incorporation into PHA-stimulated CD4:CD8 lymphocytes was 22 442 ± 2306 DPM.

Results of a representative dose response to Ba100 are shown and are expressed as % suppression of PHA-induced CD4 : CD8 lymphocyte proliferation ± SD based on the PHA-stimulated control.

Ba100 inhibited proliferation of isolated CD4 and CD8 lymphocytes (mixed at a ratio found in whole blood) in response to PHA (Figure 5). 3 dose response experiments showed that the concentration curve was linear and flat with a regression coefficient of 0.004 and SE 0.007. Ba100 at a concentration of 200 nM consistently inhibited proliferation of isolated CD4 and CD8 lymphocytes (mixed at a ratio found in whole blood) in response to PHA. The range of suppression for 3 experiments was 49.5 – 56% (mean 53.3%, median 54.5% suppression). These results suggested that processing of Ba100 by antigen-presenting phagocytes was not necessary for its immunosuppressive action.

2.5.2. Effect of Ba100 on OKT3 and fibronectin-induced proliferation of CD4 lymphocytes

Cellular adhesion involving both cell-cell and cell-extracellular matrix interactions are involved in lymphocyte trafficking and lymphocyte activation, growth and survival. Fibronectin is a key extracellular matrix protein involved in these processes (Hynes 1986; Yamada et al 1992). Cellular adhesion to fibronectin is mediated via the β_1 integrins, $\alpha_4\beta_1$ (VLA-4) and $\alpha_5\beta_1$ (VLA-5) (Guan & Hynes 1990; Hemler & Lobb 1995).

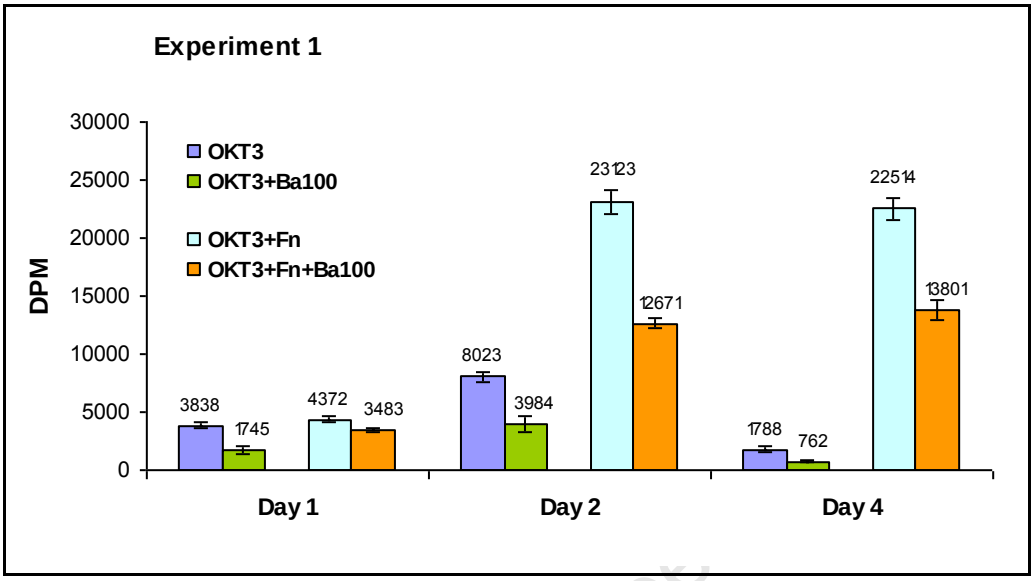
Phenotypic analysis of lymphocytes proliferating in response to PHA in the presence of Ba100 demonstrated that Ba100 downregulated the expression of the adhesion molecules CD11a, CD49d and CD29 that are important in cell-cell and cell-extracellular matrix interactions.

We next investigated the ability of Ba100 to modulate CD4 lymphocyte proliferation in response to OKT3 and fibronectin and thus potentially interfere with cell-extracellular matrix interactions.

Fibronectin has been shown to synergise with anti-CD3 antibody inducing IL-2 gene expression which promotes CD4 lymphocyte proliferation in a serum free culture system (Matsuyama et al 1989; Yamada et al 1991). Isolated CD4 lymphocytes were allowed to proliferate in the presence of OKT3 (a muromonoclonal anti-CD3 antibody) and fibronectin either alone or in combination. Proliferation of isolated CD4 lymphocytes occurred in the presence of fibronectin and immobilised OKT3. A kinetic study over 1, 2 and 4 days had shown that day 2 was the optimal time point to assess the effect of Ba100, as CD4 lymphocyte proliferation in response to OKT3 and fibronectin stimulation was maximal at this time point (data not shown).

Figure 6a-e. Effect of Ba100 on OKT3 and fibronectin-induced proliferation of CD4 lymphocytes.

a.



b.

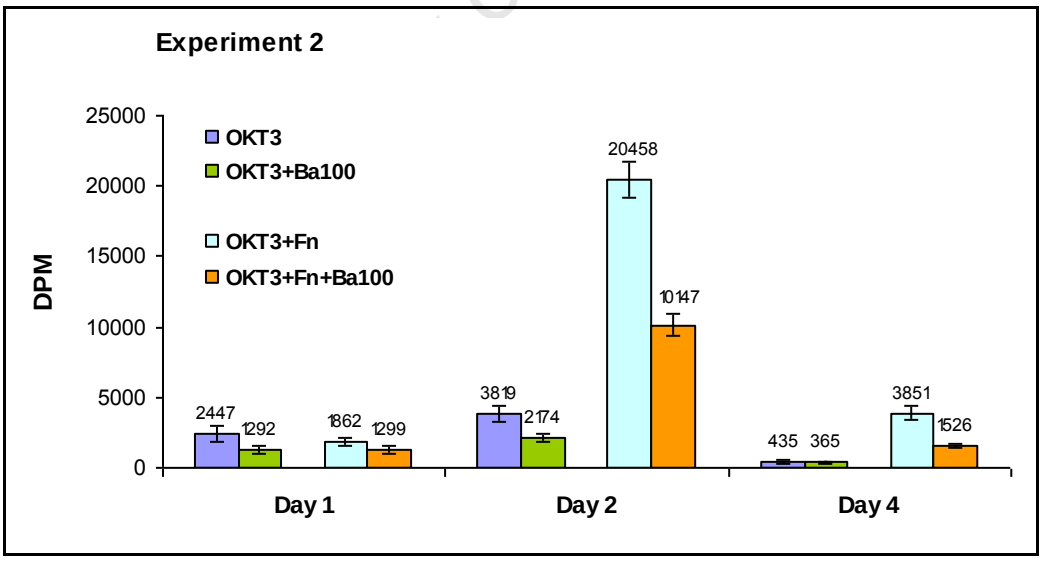
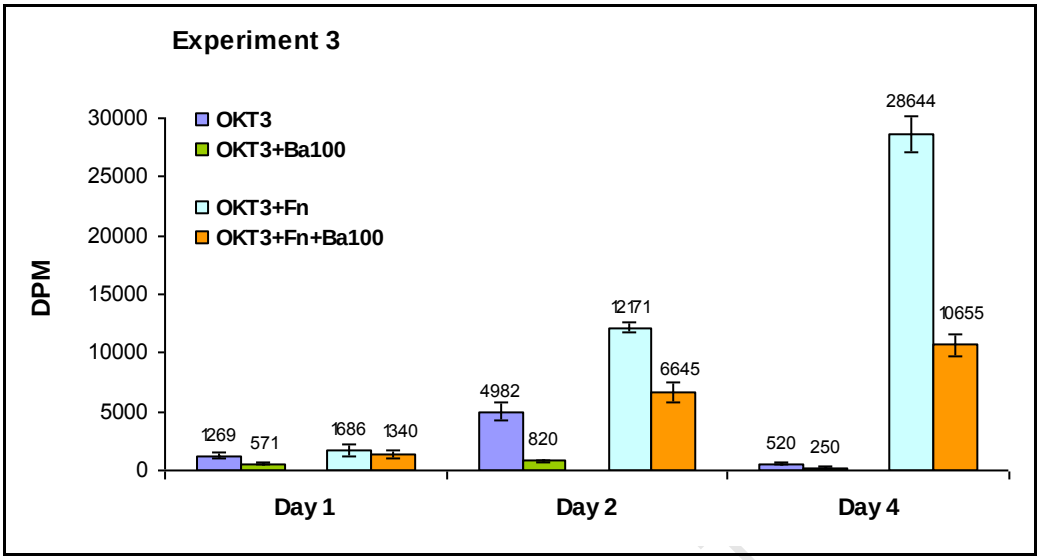


Figure 6a-e. Effect of Ba100 on OKT3 and fibronectin-induced proliferation of CD4 lymphocytes.

c.



d.

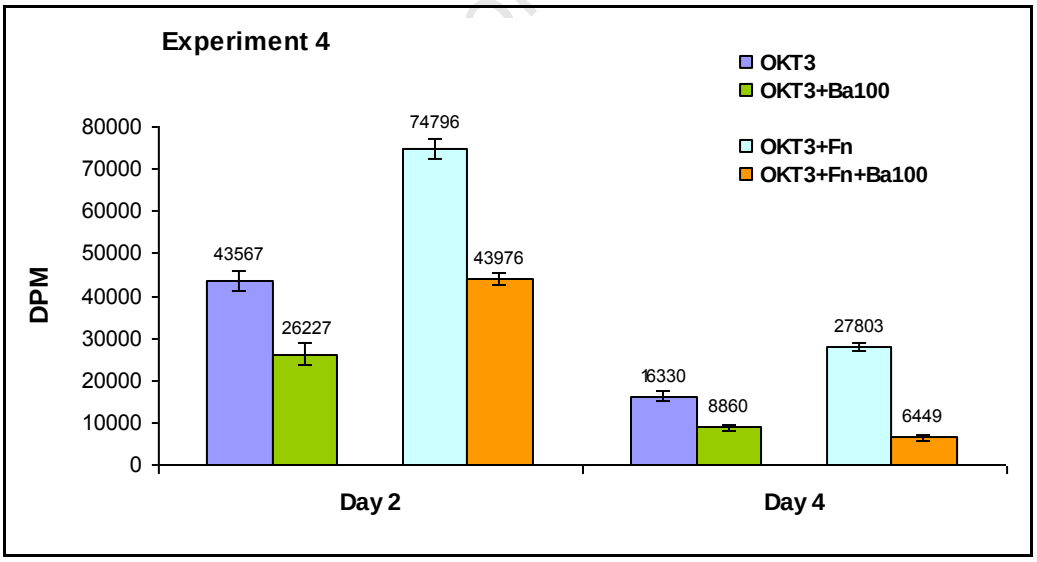
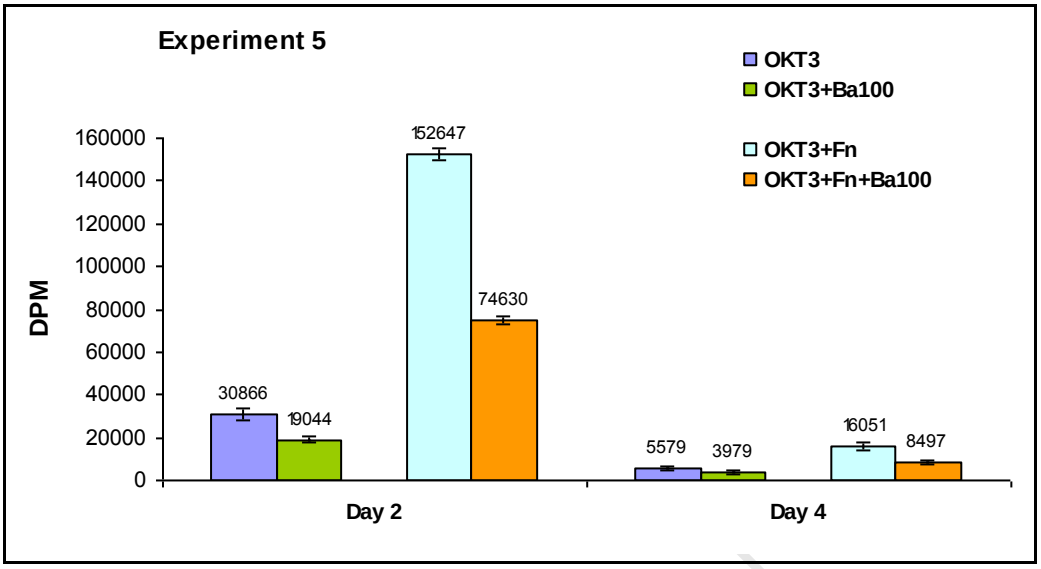


Figure 6a-e. Effect of Ba100 on OKT3 and fibronectin-induced proliferation of CD4 lymphocytes.

e.



CD4 lymphocytes were isolated from PBMCs using Dynal beads.

$1x10^5$ CD4 lymphocytes/well were incubated in OKT3-coated flat-bottomed wells in serum free culture medium (Serotec medium) with and without fibronectin (30 μg/ml) in the absence or presence of Ba100 (200 nM) at 37°C.

Ba100 was pre-incubated with CD4 lymphocytes for 30 min at 37°C before the addition of fibronectin to the wells.

[^{3}H] thymidine was added on days 1, 2 and 4 and incorporation measured 18 h later.

Background [^{3}H] thymidine incorporation for cells only, and cells + Ba100 or + fibronectin alone never averaged above 250 DPM.

Results are expressed as mean DPM of 6 replicates ± SD.

These 5 experiments indicated that Ba100 (200 nM) modulated the proliferation of isolated CD4 lymphocytes in response to OKT3 and OKT3 with fibronectin as measured by tritiated thymidine incorporation after 1, 2 and 4 days proliferation (Figure 6a-e).

Inhibition of OKT3-induced CD4 lymphocyte proliferation by Ba100 (200nM):

Day 1: Range of suppression: 47.2 – 55% (mean 52.2%, median 54.5% suppression).

Day 2: Range of suppression: 38.3 – 83.5% (mean 51%, median 43.1% suppression, optimal CD4 lymphocyte proliferation time point).

Day 4: Range of suppression: 16.1 – 57.4% (mean 39.9%, median 45.7% suppression).

Inhibition of OKT3 and fibronectin-induced CD4 lymphocyte proliferation by Ba100 (200nM):

Day 1: Range of suppression: 20.3 – 30.2% (mean 23.7%, median 20.5% suppression).

Day 2: Range of suppression: 41.2 – 51.1% (mean 46.7%, median 45.4% suppression, optimal CD4 lymphocyte proliferation time point).

Day 4: Range of suppression: 38.7 – 76.8% (mean 57.1%, median 60.4% suppression).

Ba100 was able to inhibit CD4 lymphocyte proliferation stimulated via the TCR/CD3 receptor pathway. These results also suggest that Ba100 is able to inhibit the synergistic interaction of the fibronectin receptor with TCR/CD3 mediated CD4 lymphocyte activation and possibly inhibit IL-2 production needed for proliferation.

2.6. Effect of Ba100 on PBMC proliferation in response to anti-CD28 antibody and a phorbol ester, PMA

Our data, so far, indicated that Ba100 inhibits calcium-dependent T lymphocyte proliferation pathways involving TCR/CD3 interactions with mitogen, antigen and allo-antigen, as well as T cell receptor/integrin receptor stimulated proliferation.

We then investigated whether Ba100 could inhibit calcium-independent proliferation pathways such as proliferation in response to anti-CD28 antibody and a phorbol ester, phorbol 12-myristrate 13-acetate (PMA) that directly stimulates protein kinase C. CD28 stimulation alone does not stimulate resting T cells, but when combined with PMA, T lymphocyte proliferation is induced (June et al 1994).

Table 9. Effect of Ba100 on PBMC proliferation in response to anti-CD28 antibody and PMA.

Experiment	[^{3}H]Thymidine incorporation (DPM)	Suppression of PBMC proliferation
PMA	44 431 ± 1145	-
PMA + anti-CD28	88 414 ± 2204	-
PMA + anti-CD28 + Ba100	81 341 ± 2196	7.9% ± 1.41

PBMC ($0.5x10^6$cells/well) were incubated in 1% BSA/PBS with PMA (1 ng/ml); PMA + anti-CD28 antibody (1 ng/ml) or PMA + anti-CD28 antibody + Ba100 (200 nM) at 37°C.

Ba100 was preincubated with PBMC for 30 minutes at 37°C prior to the addition of PMA or PMA + anti-CD28.

At 72 h, [^{3}H] thymidine was added and incorporation measured 18 h later.

Results of a representative experiment are shown and are expressed as mean DPM of 6 replicates ± SD or % suppression of PBMC proliferation to anti-CD28 antibody/PMA ± SD.

Ba100 at a concentration of 200 nM did not inhibit PBMC proliferation in response to anti-CD28 antibody and PMA (Table 9). This was reproducible in 4 experiments.

2.7. Effect of Ba100 on processes involved in T lymphocyte proliferation

Our data indicated that Ba100 inhibited calcium-dependent, but not calcium-independent T lymphocyte proliferation pathways. In addition, Ba100 must be added within 2 hours of initiation of lymphocyte proliferation in order to exert its inhibitory effect, suggesting that Ba100 acts early in the T cell activation pathway leading to proliferation.

To obtain further insight into the action of Ba100, we investigated the effect of Ba100 on:

1. Cytokine production during PBMC stimulation with PMA and ionomycin.
2. The ability of exogenous IL-2 to reverse the inhibition of PHA-induced PBMC proliferation in the presence of Ba100.
3. Cell cycle progression during PHA-induced PBMC proliferation.
4. Proliferating cell nuclear antigen expression.

2.7.1. Effect of Ba100 on cytokine production

Within 2 – 4 hours of activation, T cells begin to transcribe genes encoding for the cytokines IL-2, IL-3, IL-4, IFN-γ, GM-CSF and TNF-α.

PBMC proliferation in response to mitogens, antigens and alloantigens is dependent on IL-1, IL-2, IL-4 and IL-10 as well as IFN-γ production by antigen-presenting cells and lymphocytes. These cytokines are produced transiently following cell activation and act locally as autocrine, paracrine or juxtacrine regulators by binding to specific high affinity receptors on the cells that they regulate.

We have shown that Ba100 inhibits T lymphocyte proliferation and inhibits the expression of activation markers including IL-2R expression. This suggests that Ba100 may affect the generation of cytokines required for full activation and lymphocyte proliferation.

We investigated the effect of Ba100 on lymphocyte intracellular production of the cytokines IL-2, IFN-γ, and TNF-α (Th1 cytokines) and the Th2 cytokine, IL-4. We used flow cytometry to identify lymphocytes producing cytokines in response to stimulation with PMA and ionomycin (a calcium ionophore that elevates intracellular calcium). These two agents act together, bypassing the TCR to stimulate the transcription factor NF-kB, which is an essential step for the activation of cytokine producing genes (Truneh et al 1985).

i) Kinetics of lymphocyte intracellular cytokine production in response to PMA and ionomycin stimulation

The initial study investigated the optimal time point to measure intracellular cytokines.

The number of cells expressing the cytokines IFN-γ, TNF-α and IL-2 were measured as well as the level of expression of cytokines per cell at 4, 6 and 24 h.

Table 10. Kinetic study of intracellular cytokine production by PBMC in response to PMA and ionomycin.

Intracellular cytokine measured	4 h		6 h		24 h	
	% Positive cells	MFI	% Positive cells	MFI	% Positive cells	MFI
IFN-γ	38	2.4	44	1.4	20	0.34
TNF-α	48	1.8	33	0.93	24	0.72
IL-2	24	2.8	3	0.65	2	0.21

PBMC ($20x10^6$/20 ml RPMI 1640/10% AB serum) were incubated in 30ml tissue culture flasks with PMA (50 ng/ml), ionomycin (1 μm) and 2.5 μg/ml Brefeldin (inhibitor of intracellular protein transport processes) in the absence or presence of Ba100 (200 nM) for 4, 6 and 24 h at 37°C.

The cells were harvested at 4, 6 and 24 h, concentration adjusted to $10x10^6$/ml RPMI 1640/10% AB serum and 100 μl aliquots of cells were fixed with 4% paraformaldehyde, permeabilised with 0.1% saponin and then labeled with antibodies directed to the indicated cytokines (IFN-γ, TNF-α amd IL-2) and single colour analysis of cytokine antibody binding was done by flow cytometry on 10 000 CD3+ gated lymphocytes.

Results of a representative experiment are shown and are expressed as % of cells in the CD3+ lymphocyte gate expressing the cytokine and the MFI of the cytokine antibody binding.

This kinetic study indicates that the production of the intracellular cytokines IFN-γ, TNF-α and IL-2 was sufficient at 4 hours to assess modulation by Ba100 (Table 10).

2.7.2. Effect of Ba100 on intracellular IL-2 production by lymphocytes in response to PMA and ionomycin (4 h stimulation)

Four experiments were performed to investigate the effect of Ba100 on intracellular IL-2 expression by lymphocytes when stimulated with PMA and ionomycin.

Table 11. Effect of Ba100 on lymphocytes expressing and production of intracellular IL-2 in response to PMA and ionomycin (4h stimulation).

Experiment	-Ba100		+Ba100 (200 nM)		% Change (+ Ba100)	
Intracellular cytokine measured	% Positive cells	MFI	% Positive cells	MFI	% Positive cells	MFI
1. IL-2	25	8.7	2.8	1.9	88.8% ↓	78.2% ↓
2. IL-2	26	6.4	2.0	2.5	92.3% ↓	60.9% ↓
3. IL-2	28	9.2	6.9	3.3	75.4% ↓	64.1% ↓
4. IL-2	26	7.2	5.6	2.5	78.5% ↓	65.3% ↓

PBMC ($20x10^6$/20 ml RPMI 1640/10% AB serum) were incubated in 30ml tissue culture flasks with PMA (50 ng/ml), ionomycin (1 μm) and 2.5 μg/ml Brefeldin (inhibitor of intracellular protein transport processes) in the absence or presence of Ba100 (200 nM) for 4 h at 37°C.

The cells were harvested at 4 h, concentration adjusted to $10x10^6$/ml RPMI 1640/10% AB serum and 100 μl aliquots of cells were fixed with 4% paraformaldehyde, permeabilised with 0.1% saponin and then labeled with antibodies to IL-2 and single colour analysis of anti-IL-2 antibody binding was done by flow cytometry on 10 000 CD3+ gated lymphocytes.

Results of 4 experiments are shown and are expressed as % of cells in the CD3+ lymphocyte gate expressing IL-2 and the MFI of the cytokine antibody binding.

The change in intracellular IL-2 expression in the presence of Ba100 is expressed as a % of that in the absence of Ba100. ↓ indicates a decrease from that in the absence of Ba100.

These experiments (Table 11) indicated that within 4h, Ba100 at a concentration of 200 nM inhibited:

i) the generation of activated lymphocytes expressing intracellular IL-2 (range of inhibition 75.4 – 92.3%, median 83.7%).

ii) the level (MFI) of lymphocyte intracellular IL-2 expression (range of MFI inhibition 64.1 – 78.2%, median 64.7%).

2.7.3. Effect of Ba100 on intracellular IFN-γ, TNF-α and IL-4 production by lymphocytes in response to PMA and ionomycin (4 h stimulation)

A further set of 3 experiments was performed to investigate the effect of Ba100 on lymphocyte intracellular IFN-γ, TNF-α and IL-4 production in response to PMA and ionomycin.

Table 12. Effect of Ba100 on lymphocytes expressing and production of intracellular IFN-γ, TNF-α and IL-4 in response to PMA and ionomycin (4h stimulation).

Experiment	-Ba100		+Ba100 (200 nM)		% Change (+ Ba100)	
Intracellular cytokine measured	% positive cells	MFI	% positive cells	MFI	% positive cells	MFI
1. IFN-γ	36	2.25	8.5	1.23	76.4% ↓	45.3% ↓
TNF-α	48	1.86	12	0.97	75.0% ↓	47.8% ↓
IL-4	28	2.21	6.0	0.77	78.6% ↓	65.2% ↓
2. IFN-γ	38	2.18	10	1.1	73.7% ↓	49.5% ↓
TNF-α	40	1.49	13	0.83	67.5% ↓	44.3% ↓
IL-4	29	2.25	5	0.81	82.7% ↓	64.0% ↓
3. IFN-γ	39	2.24	9.8	1.1	74.9% ↓	50.9% ↓
TNF-α	42	1.55	7.3	0.85	82.6% ↓	45.2% ↓
IL-4	27	1.6	6.5	0.59	75.9% ↓	63.1% ↓

PBMC ($20x10^6$/20 ml RPMI 1640/10% AB serum) were incubated in 30 ml tissue culture flasks with PMA (50 ng/ml), ionomycin (1 μm) and 2.5 μg/ml Brefeldin (inhibitor of intracellular protein transport processes) in the absence or presence of Ba100 (200 nM) for 4 h at 37°C.

The cells were harvested at 4 h, concentration adjusted to $10x10^6$/ml RPMI/10% AB serum and 100 μl aliquots of cells were fixed with 4% paraformaldehyde, permeabilised with 0.1% saponin and then labeled with antibodies directed to the indicated cytokines (IFN-γ, TNF-α and IL-4) and single colour analysis of cytokine antibody binding was done by flow cytometry on 10 000 CD3+ gated lymphocytes.

Results of 3 experiments are shown and are expressed as % of cells in the CD3+ lymphocyte gate expressing the cytokine and the MFI of the cytokine antibody binding.

The change in intracellular cytokine expression in the presence of Ba100 is expressed as a % of that in the absence of Ba100. ↓ indicates a decrease from that in the absence of Ba100.

Ba100 at a concentration of 200 nM inhibited the generation of activated lymphocytes expressing the intracellular cytokines (Table 12):

i) IFN-γ: range of inhibition 73.7 – 76.4%, median 74.9% inhibition.

ii) TNF-α: range of inhibition 67.5 – 82.6%, median 75% inhibition.

iii) IL-4: range of inhibition 75.9 – 82.7%, median 78.6% inhibition.

There was also a decrease in the level of intracellular expression (MFI) of:

i) IFN-γ per cell: range of inhibition 45.3 – 50.9%, median 49.5% inhibition.

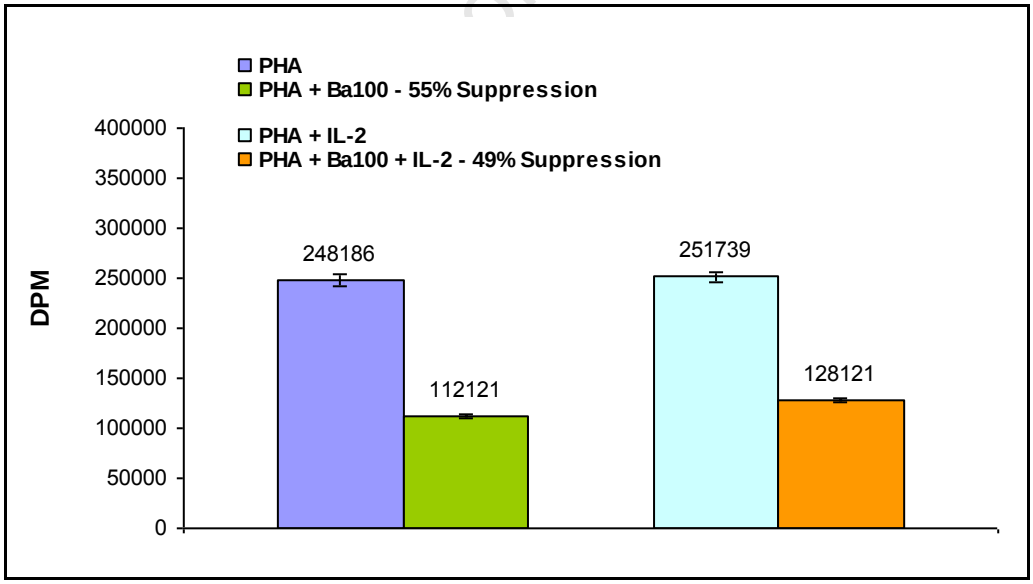
ii) TNF-α per cell: range of inhibition 44.3 – 47.8%, median 45.2% inhibition.

iii) IL-4 per cell: range of inhibition 63.1 – 65.2%, median 64% inhibition.

2.7.4. Effect of addition of exogenous IL-2 on PHA-induced PBMC proliferation in the presence of Ba100

Ba100 was shown to inhibit both Th1 and Th2 cytokines. We then looked at whether the addition of exogenous IL-2 could reverse the inhibitory action of Ba100 on PHA-stimulated lymphocytes or whether other pathways besides cytokines were involved in the immunomodulation.

Figure 7. Effect of addition of exogenous IL-2 on PHA-induced PBMC proliferation in the presence of Ba100.



PBMC (1x10^{5}cells/well) were incubated with PHA (0.00575 mitogenic units); PHA + Ba100 (200 nM); PHA + IL-2 (10 U/ml) and PHA + Ba100 + IL-2 at 37°C.

Ba100 was pre-incubated with PBMC for 30 min at 37°C prior to the addition of PHA or PHA + IL-2.

At 72 hours, [^{3}H] thymidine was added and incorporation measured 18 h later.

Background [^{3}H] thymidine incorporation into PBMC in the absence of PHA, Ba100 and IL-2 was 310 ± 35 DPM.

Results of a representative experiment are shown and are expressed as mean DPM of 6 replicates ± SD or % suppression of PBMC proliferation ± SD.

In the continual presence of Ba100, the addition of exogenous IL-2 was unable to reverse the inhibitory effect of Ba100 (200 nM) on PHA-stimulated lymphocyte proliferation (Figure 7). This was confirmed in 2 further experiments ($p = 0.4$ using the Mann-Whitney Non-parametric test). This suggests that Ba100 not only inhibits cytokine production, but also interferes with other pathways involved in mitogen-induced T lymphocyte proliferation such as engagement or expression of the IL-2 receptor. This goes together with our finding that Ba100 inhibits not only the generation of activated lymphocytes expressing the IL-2 receptor, but also downregulated IL-2 receptor expression per cell.

2.7.5. Effect of Ba100 on the distribution of PHA-stimulated PBMCs in the various phases of the cell cycle

Ba100 inhibited the induction of cytokines that play an important role in enabling T lymphocytes to move from a resting G0 phase to the activated G1 phase. We therefore investigated the effect of Ba100 on cell cycle progression.

i) The distribution of PHA-stimulated PBMCs in the various phases of the cell cycle

Two initial experiments were set up without Ba100 to determine the percentage of cells in the G_0G_1, S and G_2M phases at 24 and 48 h in response to PHA stimulation. At 24 h, most of the cells were in the G_0G_1 phase with minimal progression into the S and G_2M phases. By 48 h, cell cycling had progressed with cells now being detectable in the S and G_2M phases (Table 13).

Table 13. Distribution of PHA-stimulated PBMCs in the various phases of the cell cycle (24 and 48 h stimulation).

Experiment	Phase	% Cells per cell cycle phase	
		24 hr	48 hr
Experiment 1	G_0G_1	92	57
	S	2	23
	G_2M	2	14
Experiment 2	G_0G_1	93	56
	S	3	15
	G_2M	2	24

PBMC ($1x10^6$ cells/ml RPMI 1640/10% AB serum) stimulated with PHA in 30ml tissue culture flasks in the absence or presence of Ba100 (200 nM) were harvested at 24 and 48 hours and resuspended at $5x10^6$cells/ml RPMI 1640/10% AB serum.

Cells were stained with propidium iodide (PI) for DNA quantitation by FACS.

Cells were permeabilised with non-ionic detergent and treated with PI and RNAse.

Single parameter histograms using FL detector 3 (emission spectrum peaks at 650 nm) were used to assess results.

ii) The effect of Ba100 on the distribution of PHA-stimulated lymphocytes in the various phases of the cell cycle after 24, 40 and 48 h stimulation

At 24 h, in the absence of Ba100, the majority of cells are in the G_0G_1 phase. The addition of Ba100 (200 nM) did not accelerate cell cycle progression into the S and G_2M phases (Table 14).

Table 14. Effect of Ba100 on the distribution of PHA-stimulated PBMC in the various phases of the cell cycle (24 h stimulation).

Stimulation	Phase	% cells per cell cycle phase	
		-Ba100	+ Ba100 (200 nM)
Experiment 1	GoG_1	92	94
24 hours	S	2	1
	G_2M	2	1
Experiment 2	GoG_1	93	96
24 hours	S	3	2
	G_2M	2	1

Table 15. Effect of Ba100 on the distribution of PHA-stimulated PBMC in the various phases of the cell cycle (40 and 48 h stimulation).

Stimulation	Phase	% Cells per cell cycle phase		% Change
		-Ba100	+ Ba100 (200 nM)	
Experiment 1	G_0G_1	57	71	24.6% ↑
40 hours	S	23	13	43.5% ↓
	G_2M	14	8	42.9% ↓
Experiment 2	G_0G_1	54	64	18.5% ↑
40 hours	S	17	13	23.5% ↓
	G_2M	25	20	20.0% ↓
Experiment 3	G_0G_1	43	54	25.6% ↑
40 hours	S	28	20	28.6% ↓
	G_2M	22	18	18.2% ↓
Experiment 4	G_0G_1	52	66	26.9% ↑
40 hours	S	21	12	42.9% ↓
	G_2M	22	19	13.6% ↓
Experiment 5	G_0G_1	29	44	51.7% ↑
48 hours	S	45	27	40.0% ↓
	G_2M	21	17	19.0% ↓
Experiment 6	G_0G_1	33	51	54.5% ↑
48 hours	S	41	31	24.4% ↓
	G_2M	20	14	30.0% ↓
Experiment 7	G_0G_1	49	69	40.8% ↑
48 hours	S	33	19	42.4% ↓
	G_2M	17	10	41.2% ↓

PBMC ($1x10^6$ cells/ml RPMI 1640/10% AB serum) stimulated with PHA in 30ml tissue culture flasks in the absence or presence of Ba100 (200 nM) were harvested at 40 and 48 hours and resuspended at $5x10^6$cells/ml RPMI 1640/10% AB serum.

Cells were stained with propidium iodide (PI) for DNA quantitation by FACS.

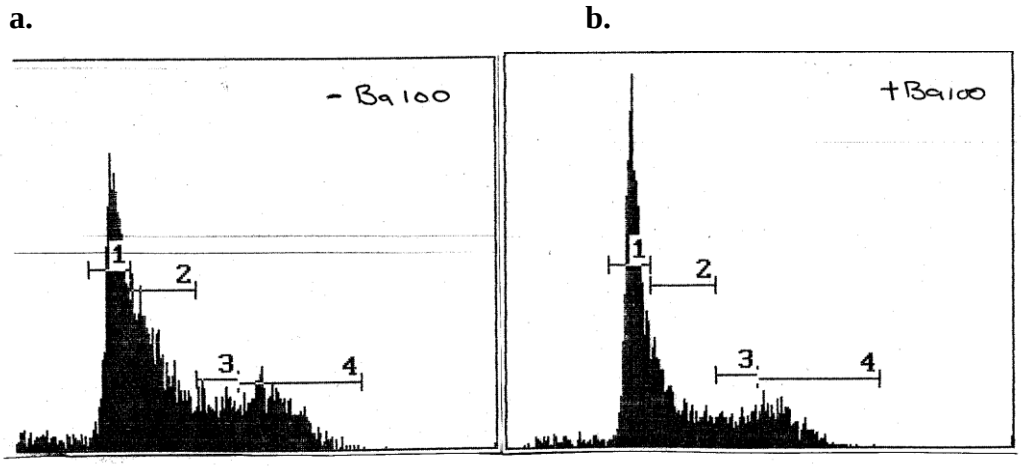
Cells were permeabilised with non-ionic detergent and treated with PI and RNAse.

Single parameter histograms using FL detector 3 (emission spectrum peaks at 650 nm) were used to assess results.

The change in the distribution of cells in the various cell cycle phases are expressed as a % of that in the absence of Ba100. ↓ indicates a decrease from in the absence of Ba100 and ↑ indicates an increase from that in the absence of Ba100.

At 48 h, Ba100 (200 nM) altered the distribution of cells in the various phases of the cell cycle. A consistent increase in the cells in the G_0G_1 phase and a decrease in the number of cycling cells, i.e. those cells in the S and G_2M cell cycle phases, was observed when Ba100 was present (Table 15 and Figure 8a and b). Ba100 inhibits PHA-stimulated lymphocyte proliferation and this was associated with inhibition of cell cycle progression from the G_0G_1 phase into the S phase.

Figure 8a and b. Effect of Ba100 on the distribution of PHA-stimulated PBMC in the various phases of the cell cycle at 48 h.



Phases of the cell cycle at 48 h: G_0G_1 phase (1 and 2), S phase (3) and G_2/M phase (4) in the absence of Ba100 (Fig. 8a) and in the presence of Ba100 (Fig. 8b).

2.7.6. Effect of Ba100 on proliferating cell nuclear antigen (PCNA) expression by PHA-stimulated PBMCs

Proliferating cell nuclear antigen is involved in DNA replication and is a critical requirement for cell cycle progression into the S phase. PCNA is first expressed through de novo protein synthesis in the mid G_1 phase, peaks in the S phase and is weakly expressed in the G_2 and M phases of the cell cycle.

We investigated whether Ba100 altered PCNA expression.

Ba100 (200 nM) inhibited the generation of cells expressing PCNA (range of suppression 21.6 – 28.3%, median 25.1% suppression) as well as the extent of PCNA expression per cell (range of suppression 27.6 – 65.5%, median 49.8% suppression) in response to PHA stimulation (Table 16).

Table 16. Effect of Ba100 on PCNA expression by PHA-stimulated PBMC (48 h stimulation).

Experiment	-Ba100		+ Ba100 (200 nM)		% Change	
	% Positive cells	MFI	% Positive cells	MFI	% Positive cells	MFI
1	68	2.9	52	2.1	23.5% ↓	27.6% ↓
2	53	2.1	38	1.0	28.3% ↓	52.4% ↓
3	60	1.7	44	0.9	26.7% ↓	47.1% ↓
4	51	2.9	40	1.0	21.6% ↓	65.5% ↓

PBMC ($1x10^6$ cells/ml RPMI 1640/10% AB serum) stimulated with PHA in 30ml tissue culture flasks in the absence or presence of Ba100 (200 nM) were harvested at 48 h and resuspended at $2x10^6$ cells/ml RPMI 1640/10% AB serum. Cells were permeabilised with 20μg/ml lysolecithin, fixed with 1% paraformaldehyde for 2 min and then treated on ice for 10 min with methanol, followed by incubation on ice with 0.1% NP-40 for 5 min.

The intracellular nuclei were then stained with anti-PCNA and analysed by FACS.

Results are expressed as % cells expressing PCNA or as MFI which represents the extent of PCNA expression per cell. The change in PCNA expression in the presence of Ba100 is expressed as a % of that in the absence of Ba100. ↓ indicates a decrease from that in the absence of Ba100.

3. DISCUSSION

Ba100, a novel C-type lectin has been isolated from the venom of the Western Cape, South African puff adder, *Bitis arietans*. Ba100, a protease with fibrinogenase activity inhibits platelet aggregation in platelet-rich plasma and clot formation in whole blood. This is due to its fibrinogenolytic activity that cleaves the Aα and Bβ chain preventing fibrinogen crosslinking and fibrin clot formation (Jennings et al 1999).

C-type lectins are known to play an important role in direct defense, cell trafficking and immune regulation (Zelensky & Gready 2005) and plant lectins such as Phytohaemagglutinin (PHA) and Concanavalin A (Con A) are well known stimulators of lymphocyte proliferation. (Vermot Desroches et al 1991; Komada et al 1996; Barten et al 2001).

In view of its C-type lectin structure and its protease/fibrinogenase activity on fibrinogen, a multifunctional protein interacting with a variety of cells, we postulated that Ba100 might have immunomodulatory potential and investigated its effect of T lymphocyte proliferation.

This study clearly shows that Ba100 consistently suppresses in a dose-dependent manner, lymphocyte proliferation in response to mitogen (IC50 ± 169 nM), antigen (IC50 ± 53 - 95 nM depending on the antigen) and alloantigen (IC50 ± 149 nM) stimulation. Curve estimation (SPSS) was used to analyse the relationship between the concentration of Ba100 and the % suppression of lymphocyte proliferation in response to mitogen, antigens and alloantigens: the concentration curves for the mitogen PHA and the MLR were simple linear and for the antigens PPD, Tetanus toxoid and Varidase, the concentration curves were linear with logarithmic transformation.

Ba100 was not lymphotoxic as the PBMCs remained viable despite prolonged culture in the presence of Ba100. Viability was confirmed microscopically at the end of each study by trypan blue exclusion or by the exclusion of propidium iodide where the effect of Ba100 on the cell cycle in response to PHA was investigated. Pre-incubation with Ba100 was not required for its anti-proliferative effects as the suppression of proliferation was the same with no pre-incubation, 30 minutes or 60 minutes pre-incubation. If Ba100 was washed away after a 30 minute pre-incubation period prior to the addition of PHA, the inhibitory effect of Ba100 was lost. These results suggested that Ba100 did not interfere with any early plasma membrane associated events in T cell activation. For standardization purposes, a 30 minute pre-incubation period was used in all the experiments. The inhibitory effect of Ba100 on PHA-induced PBMC proliferation was not achieved, if Ba100 was added 2 hours after the initiation of PBMC proliferation. This suggested that Ba100 interferes with early events of T cell activation prior to DNA synthesis and cell division.

The addition of exogenous IL-2 to activated T cells did not reverse the inhibitory effects of Ba100 on PHA-induced PBMC proliferation, suggesting that Ba100 interferes with mitogen-induced T cell proliferation at more than one site.

Having established that Ba100 did possess immunosuppressive properties with respect to mitogen and antigens, we next looked at an *in vitro* transplant model, the primary mixed lymphocyte reaction. The primary mixed lymphocyte reaction involves the generation of antigen-specific cytotoxic T lymphocytes (CTLs) and the production of cytokines which participate in delayed-type hypersensitivity responses. The generation of an immune response to an allograft or cell surface antigen is a complex process requiring interaction and collaboration of a variety of cell populations. Inhibition at any

stage of this complex cascade would lead to inhibition of lymphocyte proliferation. The primary mixed lymphocyte reaction involves a number of significant steps in addition to antigen recognition which include:

i) presentation of antigen by macrophages with subsequent production and release of IL-1 by macrophages.

ii) activation of CD4 lymphocytes with production and release of IL-2 and other cytokines (IL-1 augments the production of IL-2).

iii) IL-18 secretion and the induction of expression of ICAM-1, B7.1 (CD80), B7.2 (CD86) on monocytes (Morichika et al 2003).

iv) activation of precursor CTLs with acquisition of a receptor for IL-2 (Hayry & Defendi 1970).

v) clonal amplification and activation of CTLs by IL-2 (Hayry & Defendi 1970; Hodes & Svedmyr 1970).

vi) activation of suppressive regulatory T lymphocytes which downregulate the alloimmune response (Hirschberg & Thorsby 1977).

The IL-2/IL-2 receptor pathway is a critical rate-limiting step within the allo-immune response. Its primary effect being to amplify effector function, especially the clonal expansion of antigen-specific CTLs.

Ba100 inhibited the primary mixed lymphocyte reaction in a dose-dependent manner. Effector cells generated in a primary MLR in the presence of Ba100 exerted a suppressive effect on the proliferation of a fresh MLR. This suppressive activity was not antigen specific. Ba100 also inhibited the cytolytic activity of effector cells generated in a primary MLR.

After establishing the inhibitory effects of Ba100 on lymphocyte proliferation, we used flow cytometry to look at the effect of Ba100 on lymphocyte activation markers, co-stimulatory molecules, cell adhesion molecules and lymphocyte subsets.

Phenotypic analysis of lymphocytes after PHA-induced PBMC proliferation in the presence of Ba100 consistently showed:

i) impaired generation of activated lymphocytes as measured by the number of cells expressing HLA-DR and CD25, the marker for the IL-2 receptor, as well as a decreased number of HLA-DR and IL-2 receptors expressed per cell.

ii) impaired generation of lymphocytes expressing the costimulatory molecule CD28 as well as a decreased number of CD28 molecules expressed per cell.

iii) impaired generation of lymphocytes expressing the adhesion molecules CD11a, CD49d and CD29 as well as a decreased number of CD11a, CD49d and CD29 receptors expressed per cell.

iv) impaired generation of cytotoxic T cells expressing the S6F1 marker that is found on the killer effector cells and is induced on CD8 cells during the mixed lymphocyte reaction and a decreased number of S6F1 receptors expressed per cell.

The inhibited generation of lymphocytes expressing CD49d and CD29 molecules as well as the decreased cellular expression of these molecules suggests that memory cell adhesion to the vascular endothelium would be impaired.

Phenotypic analysis of Ba100-treated effector cells generated in a six day primary MLR also confirmed that Ba100 inhibited the generation of activated lymphocytes expressing HLA-DR and the IL-2 receptor. There was also impaired generation of natural killer cells as measured by the number of cells expressing either CD16 or CD56. Ba100 in the MLR increased the CD4:CD8 ratio possibly due to CD8 lymphocytes being unable to proliferate as a result of a decreased expression of the IL-2 receptor. Ba100 had no effect on B cells. Thus, phenotypic analysis demonstrated that Ba100 affected critical components of the alloimmune response.

T lymphocyte proliferation is dependent not only on recognition of antigen presenting Class I and Class ΙΙ receptors by lymphocyte CD8 and CD4 receptors respectively, together with the TCR/CD3 complex, but also on the interaction of antigen-presenting cells with lymphocyte accessory molecules. In addition to the antigenic signal, T cells

require a number of accessory signals for optimal activation, including molecular interactions of cell surface adhesion molecules on antigen-presenting cells (LFA-1 (CD11a/CD18), ICAM-1, VCAM-1, LFA-3) with T lymphocyte receptors (ICAM-1, LFA-1 (CD11a/CD18), VLA-4 (CD49d/CD29), CD2) together with the CD4/CD8 and CD28 co-receptors (Davis & van der Merwe 1996; Zuckerman et al 1998; Yashiro et al 1998). The CD28:B7 co-stimulatory pathway is particularly important and is a powerful activator of phosphatydilinositol 3-kinase leading to production of IL-1, IFNγ, IL-2 and IL-10 (Lenschow et al 1996; Salomon & Bluestone 2001; Sharpe & Freeman 2002; Nel 2002).

LFA-1 (CD11a/CD18) plays a critical role in leukocyte function and trafficking (Kellersh & Kolanus 2006; Graf et al 2007; Smith et al 2007; Stanley et al 2008). LFA-1 signals bidirectionally with processing of information from extracellular ligands (outside-in signaling) and inside-out signaling of cellular activation processes, thereby influencing cellular adhesion, migration, cytokine production and proliferation (Xingyuan et al 2006; Mor et al 2007).

VLA-4 (CD49d/CD29) binds to VCAM-1 and fibronectin, mediating adhesion to activated endothelial cells and the ECM respectively (Wayner et al 1989; Elices et al 1990; Collins et al 1995; Blankenberg et al 2003). VLA-4 also interacts with the endothelial junctional adhesion molecule-2 (JAM-2) (Cunningham et al 2002b).

Both LFA-1 and VLA-4 promote the differentiation of Th1 effector lymphocytes (González-Amaro et al 2005) and play an important role in lymphocyte-endothelium reactions involved in allograft rejection. LFA-1 and VLA-4 expressed on lymphocytes bind to the endothelial adhesion molecules ICAM-1 and VCAM-1 respectively (Adams & Shaw 1994; Steeber & Tedder 2000; Pribila et al 2004; Steeber et al 2005). This results in strong adhesion of lymphocytes to the endothelial wall (Tanaka et al 1993; Taub et al 1993a,b; Adams et al 1994; Gonzalez-Amaro & Sanchez 1999; Xingyuan et al 2006). Memory cells which are a critical component of the alloimmune response have an upregulated expression of LFA-1, CD2 and VLA-4 and adhere to the endothelium and ECM via VLA-4. These lymphocyte receptors VLA-4 (CD49d/CD29) and LFA-1 (CD11a/CD18) are also involved in transendothelial migration (Elices et al 1990; Hourihan et al 1993; Gonzalez-Amaro et al 2005; Rao et al 2007). Thus, Ba100, which downregulates the expression of the adhesion molecules CD11a, CD49d and CD29,

could potentially interfere with the adhesion cascade involved in the recruitment of T lymphocytes and memory cells into allografts (Briscoe et al 1998; Fujisaki et al 1998).

The formation of the immunological synapse is the initiating step in all T lymphocyte dependent immune responses establishing functional contact between T lymphocytes and antigen-presenting cells. LFA-1/ICAM-1 interactions are important in maintaining the functional unit of the immunological synapse, the peripheral supraclavicular activation cluster (pSMAC) (Lin et al 2005). VLA-4 also plays a role in the formation of the immunological synapse, relocating to the pSMAC (Mittelbrunn et al 2004). Ba100 could thus interfere at this critical point in T cell activation. Ba100 also inhibited the generation of activated lymphocytes expressing the costimulatory molecule CD28 and downregulated CD28 expression per cell. The CD28-B7 pathway provides the important second signal required for optimal T cell activation, proliferation and differentiation into effector T cells and memory cells (Salomon & Bluestone 2001; Sharpe & Freeman 2002; Nel 2002).

Ba100 inhibited the generation of T cell subsets that play a critical role in the allo-immune response: CTL, NK cells and memory cells as well as increasing the CD4:CD8 ratio. Alloreactive memory T cells are relatively resistant to tolerance (Chalasani et al 2002; Sprent & Surh 2002). Effector memory cells require minimal costimulation signaling from B7 and CD40 on antigen-presenting cells (London et al 2000). Thus, the impaired generation of memory T cells would be potentially beneficial in inducing tolerance.

In PHA-stimulated PBMC proliferation assays, Ba100 inhibited the generation of CTLs which are responsible for lymphocyte mediated cytotoxicity, an important effector mechanism in allograft rejection (Kagi et al 1996; Liu et al 1996; Barry & Bleakley 2002; Russel & Ley 2002; Rocha et al 2003).

In the mixed lymphocyte reaction assays, Ba100 inhibited the generation of NK cells as determined by the number of cells expressing either CD16 or CD56. NK cells have been shown to infiltrate allografts shortly after transplantation before T cell infiltration (Hsieh et al 2002; McNerney et al 2006). NK cells do not require prior sensitization and are not antigen-specific. NK cells have immunomodulatory lectin-like receptors that transduce both inhibitory and activating signals to the cell and the balance between

these receptors determines NK cell activation (Ryan et al 2001). Activated NK cells express chemokine receptors CXCR1 and CXCR3 and release numerous cytokines and chemokines leading to mononuclear cell recruitment and activation, and recruitment of alloreactive T cells (Kondo et al 2000; Obara et al 2005). NK cells lyse target cells via the granzyme exocytosis pathway (Shresta et al 1995), but can also induce antibody-dependent cell cytotoxicity. NK cells have been shown to play a role in solid organ allograft rejection (Totterman et al 1989; Blancho et al 1992; Bishara et al 2001; Vampa et al 2003) and may play an important role in xenografts (Kirk et al 1993; Seebach & Waneck 1997; Manilay & Sykes 1998; Khalfoun et al 2000; Rieben & Seebach 2005; Kitchens et al 2006).

In the MLR, Ba100 constantly increased the CD4:CD8 ratio and this possibly reflects the inability of CD8 lymphocytes to proliferate due to inhibition of the IL-2/IL-2 receptor pathway.

CD4 lymphocytes play a critical role in the initiation of allograft rejection. Activated CD4 lymphocytes in both the direct and indirect pathways proliferate and secrete a variety of cytokines that drive the alloimmune response leading to allograft injury (Krensky et al 1990; Keane-Myers et al 1998; Lakkis et al 1998; O'Garra 1998). These cytokines act in an autocrine and paracrine manner as growth and activating factors for CD8 cytotoxic cells, B cells and macrophages leading to destruction of the transplanted organ by direct lysis of target cells, antibody production and delayed-type hypersensitivity mechanisms respectively (Sayegh & Turka 1998; Rocha et al 2003).

Ba100 inhibited isolated CD4 and CD8 lymphocyte proliferation in response to PHA in the absence of antigen-presenting phagocytes. This suggested that no processing of Ba100 by antigen-presenting phagocytes was required for its immunomodulatory activity. Furthermore, inhibition of CD4 lymphocyte proliferation in response to OKT3, a muromonoclonal anti-CD3 antibody and OKT3 and fibronectin was observed.

Lymphocyte-extracellular matrix (ECM) interactions play an important role in lymphocyte activation, adhesion, migration and positioning within tissue microenvironments (Hynes 1986; Shimizu et al 1990; de Sousa et al 1991; Yamada et al 1992; Hauzenberger et al 1994; Ybarrondo et al 1994; Ostergaard & Ma 1995; Hunter & Shimizu 1997). Fibronectin is a key ECM protein involved in these events and

lymphocyte/fibronectin interaction plays an important role in the alloimmune response (Coito et al 2000a). Cellular adhesion to fibronectin is mediated via the β1 integrins, α4β1 (VLA-4) and α5β1 (VLA-5) (Guan & Hynes 1990; Hemler & Lobb 1995). α4β1 is upregulated during lymphocyte proliferation. The α5β1 integrin is present on resting CD45 RA dim memory cells (Klingemann & Dedhar 1989). The adhesion of T lymphocytes to fibronectin is regulated by engagement of the antigen-specific TCR/CD3 complex and the engagement of CD2, CD7 and CD28 molecules (Shimizu et al 1992, 1995; Chan et al 1997). Chemokines such as RANTES, MCP-1 and MIP-1 also play a role in regulating integrin-mediated lymphocyte binding to fibronectin (Weber et al 1996, Carr et al 1994, 1996).

Fibronectin plays an important role in the effector phase of the alloimmune response which is dependent on the adhesion and migration of alloreactive cells into the graft (Kupiec-Weglinski et al 1993; Coito et al 1994a,b, 2000a). It has been shown that *in vivo* interactions between the lymphocyte α4β1 integrin receptor and the cell associated CS-1 motif of fibronectin are critical in the acute allograft rejection cascade (Coito et al 1998). Blockade of these interactions with CS-1 peptides prevented rejection of rat cardiac allografts by reducing intragraft infiltration of CD4 and CD8 lymphocytes, decreasing vascular expression of VCAM-1 and ICAM-I and decreasing cytokine (IL-2, IFN-γ, IL-4, IL-5 and IL-6) production (Coito et al 1998; Coito 2000a,b). Administration of CS-1 peptides to mice recipients of islet allografts prevented graft infiltration suggesting a role for α4β1/fibronectin interactions in lymphocyte homing to the graft site (Stegall et al 1999). Rat cardiac allograft models have shown that upregulation of vascular fibronectin is a common early step in both allo- and iso-transplants and is an important signal that triggers lymphocyte recruitment at the graft site (Coito et al 1994a,b, 1997). Later, following cardiac allo-transplantation, cellular fibronectin is predominantly expressed in the myocardium, but this is absent in isografts.

Thus, the ability of Ba100 to inhibit CD4 lymphocyte proliferation in response to OKT3 and fibronectin may in part relate to its ability to downregulate the CD49d and CD29 adhesion molecules and thus impair cell-extracellular matrix interactions that participate in lymphocyte activation. This is potentially advantageous in inhibiting the alloimmune response.

Ba100 inhibits lymphocyte proliferative responses which are initiated by stimuli that result in an increase in intracellular calcium concentration, but is unable to inhibit PBMC proliferation in response to anti-CD28 antibody and PMA. Growth factor stimulation with IL-2 or IL-4 or treatment with anti-CD28 monoclonal antibodies plus PMA is not associated with a rise in intracellular calcium.

These results collectively suggest that the immunosuppressive effects of Ba100 are a result of inhibition of an early step in T cell activation involving calcium-dependent activation pathways that result in the inhibition of IL-2 production and IL-2 receptor expression.

Having demonstrated that Ba100 inhibited CD4 lymphocyte proliferation, we investigated its effect on the generation of cytokines. Within 2-4 hours following activation, T cells begin to transcribe genes encoding several cytokines including IL-2, IL-3, IL-4, IFN-γ, GM-CSF and TNFα. These cytokines act locally as autocrine, paracrine and juxtacrine regulators by binding to specific high affinity receptors on the cells that they affect. Ba100 was shown to inhibit the number of activated lymphocytes expressing the IL-2 receptor and to downregulate lymphocyte IL-2 receptor expression. Ba100 inhibited the generation of Th1 cytokines (IFN-γ, TNF-α and IL-2) and the Th2 cytokine, IL-4 by activated lymphocytes. It has been shown in human T cells activated via the TCR/CD3 complex that the amount of IL-2 produced correlates closely with the level of cellular calcineurin activity (Fruman et al 1992). Ba100 at a concentration of 200 nM resulted in a mean of 67.1% inhibition of intracellular IL-2 production by activated lymphocytes. As Ba100 inhibited activated lymphocyte production of cytokines (IL-2, IL-4, IFN-γ and TNF-α) in response to stimulation by a combination of PMA and ionomycin, this suggests that Ba100 inhibits calcium-dependent activation of the transcription factor NFκβ (Truneh et al 1985), which is an essential step for the activation of cytokine producing genes.

The Th1 cytokines promote immunopathologic functions by mediating delayed-type hypersensitivity, promoting generation of cytotoxic T cells (CTLs), activating natural killer cells, activating macrophages with consequent production of nitric oxide, reactive oxygen intermediates, degradative enzymes and TNF-α production (Rocha et al 2003). IFN-γ and TNF-α also upregulate B7 costimulatory molecule expression, thereby

enhancing T cell activation (Pechhold et al 1997). IL-4 acts as a growth-promoting cytokine in CD4 Th2 cells promoting further cytokine secretion (Swain et al 1990).

Thus, Ba100 by downregulating intracellular Th1 cytokine production as well as the production of the growth-promoting cytokine, IL-4, could potentially impair the alloimmune response leading to allograft injury and phenotypic analysis has shown that Ba100 impairs the generation of CTLs and NK cells.

We next investigated the effect of Ba100 on cell cycle progression and proliferating cell nuclear antigen expression.

Engagement of the T cell receptor and activation of co-stimulatory signals such as the CD28 co-stimulatory pathway (June et al 1994) allows gene transcription and production of cytokines which enable T cells to move from a resting G0 phase to the activated G1 phase. The progression from G1 through to the S phase is regulated through a variety of receptors on the T cell surface including the IL-2 receptor. The binding of T cell growth factors such as IL-2 to high affinity receptors, initiates signaling events required for the progression of the T cell in the G1 phase into the S phase and ultimately into mitosis. The enzymes critical for the induction of cell division include cyclin/cyclin-dependant kinases and de novo purine and pyrimidine synthesis which is essential before lymphocytes can complete cell division. Ba100 consistently results in a decrease in the number of cycling cells, blocking cell cycle progression from the G0G1 into the S phase.

PCNA (proliferating cell nuclear antigen), a member of the DNA sliding camp family, plays an essential role in nucleic acid metabolism, DNA replication and repair in all eukaryotes (Kelman 1997; Jonsson & Hubscher 1997; Maga & Hubscher 2003). PCNA plays a critical role in cell cycle regulation interacting with several eukaryotic cell cycle proteins. It binds to cyclin-CDK complexes (Xiong et al 1992) as well as the CDK inhibitor p21 (Gulbis et al 1996; Knibiehler et al 1996). Each phase of the cell cycle is under control of a specific CDK-cyclin complex: CDK4,6-cyclin-D complex regulates progression through G1, CDK2-cyclin-E regulates transition from the G1 to the S phase (a Restriction point), CDK2-cyclin-A and CDK1-cyclin-A act throughout the S phase, and CDK1-cyclin-B regulates mitosis (Maga & Hubscher 2003). PCNA is a critical requirement for cell cycle progression into the S phase and is first expressed in the mid-

G1 phase (Celis & Celis 1985). PCNA expression peaks in the S phase, but continues to be weakly expressed in the G2 and M phases of the cell cycle (Celis & Celis 1985).

Specific checkpoint mechanisms can be activated, thereby halting progression through the cell cycle if necessary. PCNA is able to form complexes with all these CDK-cyclin complexes as well as with the critical check point proteins, thereby transducing both positive and negative feedback signals on the cell cycle progression (Maga & Hubscher 2003).

Ba100 was shown to prevent progression of the cell cycle from the G0G1 phase into the S phase and PCNA which is required for progression into the S phase was consistently shown to be downregulated. In addition, Ba100 inhibits production of IL-2 and downregulates IL-2 receptor expression, both of which are critical in initiating signaling events required for progression of the T cell in the G1 phase into the S phase.

In summary, we find that Ba100, a novel C-type lectin has immunomodulatory potential through its ability to inhibit the calcium-dependent activation pathways required for T lymphocyte proliferation. This is associated with a decrease in the number of lymphocytes expressing the activation markers HLA-DR and CD25 (IL-2 receptor); impaired generation of CTLs, NK cells and memory cells; decreased IL-2 receptor expression; downregulation of important adhesion/co-stimulatory molecule expression and impaired cytokine production (IL-2, IL-4, IFN-γ and TNF-α). In addition, Ba100 interrupts the cell cycle of the lymphocyte, inhibiting cell cycle progression from the G0G1 phase into the S phase and this is associated with consistent downregulation of PCNA expression.

Thus, the immunosuppressive properties of Ba100 together with its ability to inhibit platelet aggregation and thrombus formation make it a potentially useful immunosuppressive agent for both allo- and xenotransplantation.

CHAPTER SIX:

EXPERIMENTAL SECTION : MODULATION OF LYMPHOCYTE PROLIFERATION BY Ba25

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2.7. Effect of Ba25 on processes involved in T lymphocyte proliferation

2.7.1. Effect of Ba25 on IFN-γ, IL-4 and IL-10 production during the mixed lymphocyte reaction

2.7.2. Effect of addition of exogenous IL-2 on PHA-induced PBMC proliferation in the presence of Ba25

2.7.3. Effect of Ba25 on the distribution of PHA-stimulated PBMCs in the various phases of the cell cycle

2.7.4. Effect of Ba25 on proliferating cell nuclear antigen (PCNA) expression by PHA-stimulated PBMCs

2.7.5. Effect of Ba25 on lymphocyte membrane stability

i) Effect of α-tocopherol on PHA-induced PBMC proliferation in the presence of Ba25

ii) Effect of Indomethacin on PHA-induced PBMC proliferation in the presence of Ba25

3. Discussion

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CHAPTER SIX:

EXPERIMENTAL SECTION : MODULATION OF LYMPHOCYTE PROLIFERATION BY Ba25

1. INTRODUCTION

Ba25, a C-type lectin was isolated from the venom of the Western Cape, South African puff adder, *Bitis arietans*, using a GPIIbIIIa affinity column. Functional studies demonstrated that Ba25 stimulated platelet agglutination in platelet-rich plasma and inhibited clot formation in whole blood. Ba25 interacts with the GPIbα receptor of the von Willebrand receptor complex GPIb-IX-V close to the binding site of the von Willebrand factor as well as the collagen receptor $\alpha_2\beta_1$ leading to activation of the platelet fibrinogen receptor GPIIbIIIa and increased fibrinogen binding without any associated platelet activation. Ba25 also inhibited collagen-induced platelet P-selectin expression (Jennings et al 2005, Jennings unpublished data).

Platelets play an important role in inflammation, releasing pro-inflammatory factors and expressing both integrins and immunoreceptors that modulate intra/intercellular interactions, cellular adhesion and transendothelial migration (Weyrich et al 2003; Kasirer-Friede et al 2007). Activated platelets have been shown to enhance activated lymphocyte adhesion to the extracellular matrix via integrin, CD40-CD40 ligand and P-selectin-PSGL-1 mediated interactions (Shenkman et al 2006). Platelet activation is an important contributory component to the alloimmune response and allograft injury (Zhang et al 2003; 2005; Xu et al 2006).

Platelets and lymphocytes have a number of integrin receptors in common including the collagen receptor $\alpha_2\beta_1$ (Pribila et al 2004, Kasirer-Friede et al 2007). The lymphocyte $\alpha_2\beta_1$ integrin receptor mediates lymphocyte adhesion to collagen and contributes to signaling processes involving the p38 MAPK pathway as well as the PI3-K/Akt activation pathway (Heino 2000; Gendron et al 2003; Boisvert et al 2007).

We thus postulated that Ba25 which modulates platelet function, through its interaction with the platelet GPIbα and $\alpha_2\beta_1$ receptors, might modulate lymphocyte function by targeting the common integrin receptor $\alpha_2\beta_1$.

1.1. Experimental approach

We investigated the ability of Ba25 to inhibit T lymphocyte proliferation and the experimental approach was similar to that followed for Ba100. These studies indicated that although Ba25 inhibited T lymphocyte proliferation, it differed from Ba100 in that it did not modulate T lymphocyte activation markers nor cell cycle progression and it generated an immunosuppressive cytokine profile. We therefore looked at the potential effect of Ba25 on the generation of immunosuppressive prostanoids.

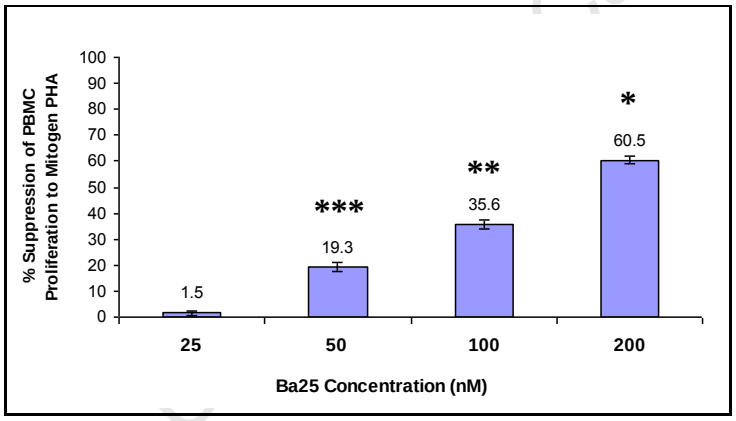
2. RESULTS

2.1. PBMC proliferation assays in response to the mitogen, PHA

i) Effect of Ba25 on PHA-induced PBMC proliferation

The effect of various concentrations of Ba25 on proliferation of PBMC in response to PHA was investigated and the data from a representative experiment is shown in Fig. 1.

Figure 1. Effect of various concentrations of Ba25 on PHA-induced PBMC proliferation.



PBMC ($1x10^5$ cells/well) were incubated with PHA (0.00575 mitogenic units) or PHA + Ba25 (200, 100, 50, 25 nM concentrations) at 37°C.

Ba25 was pre-incubated with PBMC for 30 min at 37°C prior to the addition of PHA.

At 72 h, [^{3}H] thymidine was added, incorporation measured 18 h later and results are expressed as mean DPM of 6 replicates ± SD.

Background [^{3}H] thymidine incorporation into PBMC in the absence of PHA or presence of Ba25 were 242 ± 12 DPM and 225 ± 16 DPM respectively.

[^{3}H] thymidine incorporation into PHA-stimulated PBMC was 249 316 ± 4031 DPM.

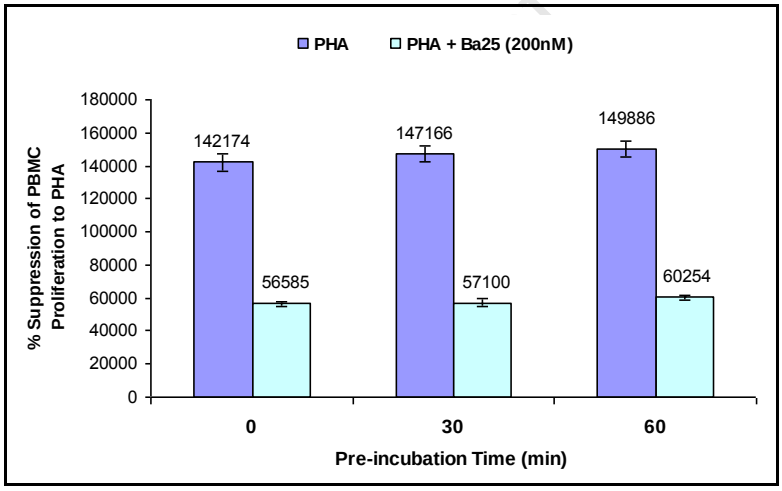
Results of a representative dose response to Ba25 are shown and are expressed as % suppression of PBMC proliferation to PHA ± SD based on the PHA-stimulated control. * $p < 0.001$, ** $p < 0.002$, *** $p < 0.0005$, comparing % suppression at each successive concentration.

In the absence of PHA, Ba25 did not alter background [^{3}H] thymidine incorporation. When Ba25 was pre-incubated with PBMC for 30 min prior to the addition of PHA, a dose-dependent inhibition of the proliferation of the PBMC was observed (Figure 1). Two further dose response experiments confirmed that the relationship between the concentration of Ba25 and % suppression of PBMC proliferation in response to PHA was linear with logarithmic transformation ($p < 0.0001$) and the IC50 was ± 138nM. A further set of 10 experiments was done to confirm the effect of a 30 min pre-incubation period of Ba25 (200 nM) on PHA-induced proliferation. For each experiment, inhibition of proliferation was observed and the range of suppression of proliferation for these 10 experiments was 42 – 76% (mean 59.8%, median 61% suppression). Exclusion of trypan blue by cells harvested at 72 h indicated that the cells were not being killed by Ba25 and thus the inhibition of proliferation was not due to cytotoxicity.

ii) Effect of pre-incubation with Ba25 on PHA-induced PBMC proliferation

We investigated whether pre-incubation with Ba25 was required for the inhibitory effect on PHA-induced PBMC proliferation.

Figure 2. Effect of pre-incubation with Ba25 on PHA-induced PBMC proliferation.



PBMC (1x10^5 cells/well) were incubated with PHA (0.00575 mitogenic units) or PHA + Ba25 (200 nM) at 37°C.
Ba25 was either pre-incubated for 30 min or 60 min with PBMC at 37°C prior to the addition of PHA or added to the PBMC when the PHA was added.
At 72 h, [^{3}H] thymidine was added and incorporation measured 18 h later.
Background [^{3}H] thymidine incorporation for cells only or cells + Ba25 (200 nM) never averaged above 375 DPM.
Results of a representative experiment are shown and are expressed as the mean DPM of 6 replicates ± SD.

We found that pre-incubation of PBMC with Ba25 was not required for its anti-proliferative effect. The percentage suppression of PHA-induced PBMC proliferation with a 30 min and 60 min pre-incubation period (61.2% ± 1.27 (SD) and 59.8% ± 1.3 (SD) suppression respectively) was the same as that when Ba25 was added at the time of addition of PHA (60.2% ± 1.2 (SD) suppression) (Figure 2). This was confirmed in two further experiments (regression coefficient 0.0001, SE 0.010). For standardization purposes, a 30 minute pre-incubation period was used in all the experiments, unless otherwise stated.

iii) Effect of Ba25 on PHA-induced PBMC proliferation when added 2 h after PHA

The ability of Ba25 to modulate PHA-induced PBMC proliferation after the stimulus for proliferation had been added, was investigated.

To assess whether Ba25 acted early or late in T cell signal transduction, Ba25 was added 2 h after the addition of the mitogenic stimulus, PHA.

Table 1. Effect of Ba25 on PHA-induced PBMC proliferation when added 2h after PHA.

Reaction		[^{3}H] Thymidine incorporation (DPM)	Suppression of PBMC proliferation to PHA
Mitogen	Ba25 (200 nM)		
PHA	-	244 433 ± 10 060	-
PHA	30 min pre-incubation	133 438 ± 2019	45.4% ± 1.2
PHA	Added 2h after PHA	247 127 ± 2684	0%

PBMC (1x10^5 cells/well) were incubated with PHA (0.00575 mitogenic units) or PHA + Ba25 (200nM) at 37°C.

Ba25 was pre-incubated with PBMC for 30 min at 37°C prior to the addition of PHA or Ba25 was added 2h after PHA.

At 72 h, [^{3}H] thymidine was added and incorporation measured 18 h later.

Background [^{3}H] thymidine incorporation into PBMC in the absence of PHA or presence of Ba25 were 201 ± 14 DPM and 221 ± 17 DPM respectively.

Results of a representative experiment are shown and are expressed as mean DPM of 6 replicates ± SD or % suppression of PBMC proliferation to PHA ± SD.

Ba25 was unable to inhibit PHA-induced PBMC proliferation if added 2 h after PHA (Table 1), suggesting that Ba25 exerted its inhibitory effect early on in T cell activation. This was reproducible in two further experiments.

iv) Effect of washing away Ba25 on PHA-induced PBMC proliferation

The effect of pre-incubating Ba25 with PBMC, then washing it away before the addition of PHA to initiate proliferation, was investigated.

Table 2. Effect of washing away Ba25 on PHA-induced PBMC proliferation.

Reaction		[^{3}H] Thymidine incorporation (DPM)	Suppression of PBMC proliferation to PHA
Mitogen	Ba25 (200 nM)		
PHA	-	152 621 ± 3775	-
PHA	30 min pre-incubation	76 347 ± 1459	49.9% ± 0.42
PHA	30 min pre-incubation, then wash	159 511 ± 3908	0%

PBMC (1x10^5 cells/well) were incubated with PHA (0.00575 mitogenic units) or PHA + Ba25 (200nM) at 37°C.

Ba25 was pre-incubated with PBMC for 30 min at 37°C prior to the addition of PHA. For one reaction Ba25 was washed away after the 30 min pre-incubation period.

At 72 h, [^{3}H] thymidine was added and incorporation measured 18 h later.

Background [^{3}H] thymidine incorporation into PBMC in the absence of PHA or presence of Ba25 were 390 ± 25 DPM and 412± 37 DPM respectively.

Results of a representative experiment are shown and are expressed as mean DPM of 6 replicates ± SD or % suppression of PBMC proliferation to PHA ± SD.

Removing Ba25 from the reaction prior to the addition of PHA resulted in a complete loss of the inhibitory effect of Ba25 on PHA-induced PBMC proliferation (Table 2). Ba25 needs to be present during the proliferation period for its inhibitory effect to be observed. This was reproducible in two further experiments.

2.1.1. Lymphocyte populations and phenotype of the lymphocytes after PBMC proliferation in response to PHA in the absence or presence of Ba25

Flow cytometry was used to analyse the phenotype of the lymphocyte population after proliferation of PBMC in response to PHA in the absence or presence of Ba25 (200 nM). Both the % of cells in the lymphocyte gate (set on lymphocyte forward and side scatter properties) expressing a particular receptor and the MFI (mean fluorescent intensity) of the antibody binding was determined. Lymphocytes in the lymphocyte gate were analysed for expression of activation markers HLA-DR and the interleukin 2 receptor (CD25); adhesion molecule CD29 and NK cell markers in the form of CD56.

Anti-CD3 was used to detect T cells. Anti-CD4 and anti-CD8 was used to determine T cell subsets.

Table 3. Lymphocyte populations and phenotype after proliferation of PBMC in response to PHA in the absence or presence of Ba25 (200 nM).

Receptors	PHA-stimulated PBMC -Ba25		PHA-stimulated PBMC + Ba25 (200 nM)		% Change + Ba25 (200 nM)	
	% Positive cells	MFI	% Positive cells	MFI	% Positive cells	MFI
CD3	85.3	5.08	69.2	3.2	18.9% ↓	37.0% ↓
CD4	39.0	10.2	30.1	5.3	22.8% ↓	48.0% ↓
CD8	33.1	48.7	36.6	54.7	10.6% ↑	No change
CD56	10.0	5.7	7.1	5.5	29% ↓	No change
CD29	91.2	6.6	78.1	5.8	14.4% ↓	No change
HLA-DR	72.0	39.9	63.0	32.2	12.5% ↓	No change
CD25	92.2	19.1	83.3	17.8	No change	No change
CD4:CD8	1.2		0.82		31.6% ↓	

PBMC ($1x10^5$ cells/well) were incubated with PHA (0.00575 mitogenic units) or PHA + Ba25 (200 nM) at 37°C.

Ba25 was pre-incubated with PBMC for 30 min at 37°C prior to addition of PHA.

At 72 h, the cells were harvested, then labeled with antibodies directed to the indicated lymphocyte receptors and single colour analysis of antibody binding was done by flow cytometry on gated lymphocytes.

Results of a representative experiment are shown and are expressed as % of cells in the lymphocyte gate expressing the receptor and the MFI (mean fluorescent intensity or number of receptors per cell) of the antibody binding. The change in these parameters in the presence of Ba25 is expressed as a % of that in the absence of Ba25. ↓ indicates a decrease from that in the absence of Ba25 and ↑ indicates an increase from that in the absence of Ba25.

A change in the % of cells expressing a specific receptor and a change in MFI in the presence of Ba25 was considered a real change if this was more than a 10% and 20% change respectively. Only real changes decided by these criteria are shown in the last 2 columns.

From the results of a representative experiment given in Table 3, it appears that Ba25 had little effect on the generation of lymphocytes expressing the activation markers HLA-DR and CD25 nor did it affect cellular expression of these receptors. Ba25 inhibited the generation of natural killer cells expressing CD56 and had minimal effect on the generation of cells expressing CD29. There was a decrease in the CD4:CD8 ratio.

These results were confirmed in 4 further experiments with PHA-induced PBMC proliferation in the presence of Ba25 (200 nM). The range of suppression and median suppression for both the percentage of cells expressing a receptor and the number of receptors expressed per cell are tabulated in Table 4.

Table 4. Change in lymphocyte populations and phenotype after proliferation of PBMC in response to PHA in the presence of Ba25 (200 nM).

Receptors	PHA-stimulated PBMC + Ba25 (200 nM)			
	% Positive cells		MFI	
	Range of suppression	Median	Range of suppression	Median
HLA-DR	12.0 – 18.4%	17.1%	16.7 – 21.4%	19.9%
CD25	4.6 – 9.8%	7.1%	6.9 – 9.6%	8.3%
CD56	16.6 – 30.1%	22.8%	4.2 – 10.9%	7.2%
CD29	6.2 – 13.6%	10.3%	7.4 – 14.3%	8.9%
CD4:CD8	28.6 – 31%	29.8%		

PBMC ($1x10^5$ cells/well) were incubated with PHA (0.00575 mitogenic units) or PHA + Ba25 (200 nM) at 37°C.

Ba25 was pre-incubated with PBMC for 30 min at 37°C prior to the addition of PHA.

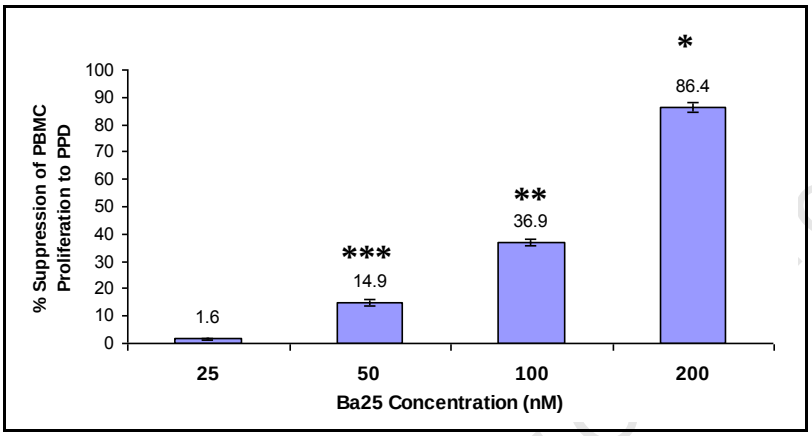
At 72 h, the cells were harvested, then labeled with antibodies directed at the indicated lymphocyte receptors and single colour analysis of antibody binding was done by flow cytometry on gated lymphocytes.

The range of suppression and the median suppression for both the % of cells in the lymphocyte gate expressing the receptor and the MFI (mean fluorescent intensity) of the antibody binding are shown.

2.2. PBMC proliferation assays in response to various antigens

The effect of Ba25 on proliferation of PBMC in response to various antigens was investigated and the data from representative experiments are shown in Figure 3a - c.

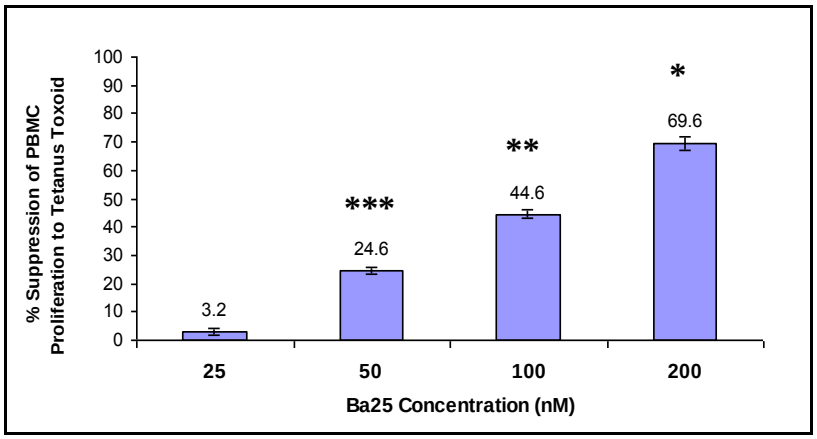
Figure 3a. Effect of various concentrations of Ba25 on PPD-induced PBMC proliferation.



PBMC (1x10^{5}cells/well) were incubated with the antigen PPD without or with Ba25 (200, 100, 50, 25 nM) at 37ºC. Ba25 was pre-incubated with PBMC for 30 min at 37ºC prior to the addition of PPD.
On day 7, [^{3}H] thymidine was added, incorporation measured 18h later and results are expressed as mean DPM of 6 replicates ± SD.
Background [^{3}H] thymidine incorporation into PBMC in the absence of PPD was 410 ± 24 DPM and in the presence of Ba25 was 394 ± 42 DPM.
[^{3}H] thymidine incorporation into PBMC in the presence of PPD was 133 615 ± 3000 DPM.
Results of a representative dose response to Ba25 are shown and expressed as % suppression of PBMC proliferation to PPD ± SD based on the PPD-stimulated control. * $p < 0.0001$,** $p < 0.005$, *** $p < 0.003$, comparing % suppression at each successive concentration.

Ba25 dose dependently (25 – 200 nM) suppressed PBMC proliferation in response to the antigen PPD (Figure 3a). Two further dose response experiments confirmed that the relationship between the concentration of Ba25 and % suppression of PBMC proliferation in response to PPD was linear with logarithmic transformation ($p < 0.0001$) and the IC50 was ±118nM.

Figure 3b. Effect of various concentrations of Ba25 on Tetanus toxoid-induced PBMC proliferation.



PBMC (1x10^{5}cells/well) were incubated with the antigen Tetanus toxoid without or with Ba25 (200, 100, 50, 25 nM) at 37ºC.

Ba25 was pre-incubated with PBMC for 30 min at 37ºC prior to the addition of Tetanus toxoid.

On day 7, [^{3}H] thymidine was added, incorporation measured 18h later and results are expressed as mean DPM of 6 replicates ± SD.

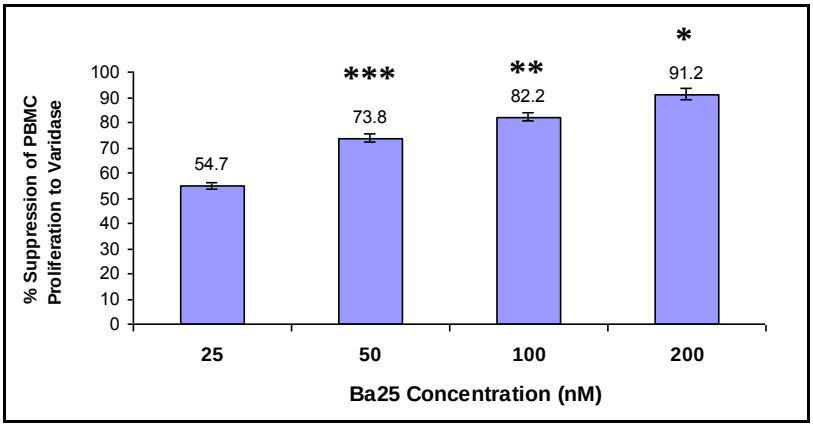
Background [^{3}H] thymidine incorporation into PBMC in the absence of Tetanus toxoid was 385 ±16 DPM and in the presence of Ba25 was 376 ± 20 DPM.

[^{3}H] thymidine incorporation into PBMC in the presence of Tetanus toxoid was 121 660 ± 5245 DPM.

Results of a representative dose response to Ba25 are shown and expressed as % suppression of PBMC proliferation to Tetanus toxoid ± SD based on the Tetanus toxoid-stimulated control. * $p < 0.001$, ** $p < 0.004$, *** $p < 0.003$, comparing % suppression at each successive concentration.

Ba25 dose dependently (25 – 200 nM) suppressed PBMC proliferation in response to the antigen Tetanus toxoid (Figure 3b). Two further dose response experiments confirmed that the relationship between the concentration of Ba25 and % suppression of PBMC proliferation in response to Tetanus toxoid was linear with logarithmic transformation ($p < 0.0001$) and the IC50 was ± 113nM.

Figure 3c. Effect of various concentrations of Ba25 on Varidase-induced PBMC proliferation.



PBMC ($1x10^5$cells/well) were incubated with the antigen Varidase without or with Ba25 (200, 100, 50, 25 nM) at 37ºC.

Ba25 was pre-incubated with PBMC for 30 min at 37ºC prior to the addition of Varidase.

On day 7, [^{3}H] thymidine was added, incorporation measured 18h later and results are expressed as mean DPM of 6 replicates ± SD.

Background [^{3}H] thymidine incorporation into PBMC in the absence of Varidase was 354 ± 28 DPM and in the presence of Ba25 was 362 ± 33 DPM.

[^{3}H] thymidine incorporation into PBMC in the presence of Varidase was 41 229 ± 1058 DPM.

Results of a representative dose response to Ba25 are shown and expressed as % suppression of PBMC proliferation to Varidase ± SD based on the Varidase-stimulated control. * $p < 0.14$, ** $p < 0.018$, *** $p < 0.005$, comparing % suppression at each successive concentration.

Ba25 dose dependently (25 – 200 nM) suppressed PBMC proliferation in response to the antigen Varidase (Figure 3c). Two further dose response experiments confirmed that the relationship between the concentration of Ba25 and % suppression of PBMC proliferation in response to Varidase was linear with logarithmic transformation ($p < 0.0005$) and the IC50 was ± 20nM.

A further set of 2 experiments with each antigen was done which confirmed the inhibitory effect of a 30 min pre-incubation period of Ba25 (200 nM) on antigen-stimulated proliferation. The range of suppression of :

i) PPD-induced proliferation was 85-90% (mean 87.8%, median 87.5% suppression).

ii) Tetanus toxoid-induced proliferation was 68-72% (mean 70%, median 70% suppression).

iii) Varidase-induced proliferation was 90-92% (mean 91%, median 91% suppression).

2.3. PBMC proliferation to alloantigens in a primary mixed lymphocyte reaction (MLR)

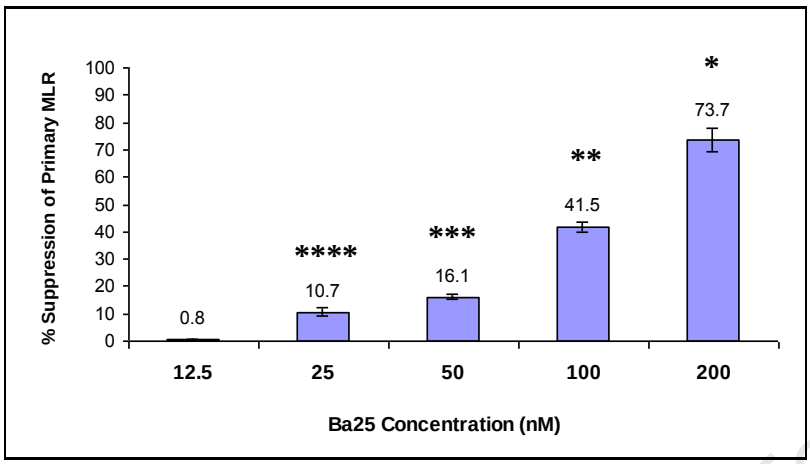
The immunosuppressive properties of Ba25 on T lymphocyte responses to mitogen and antigens was encouraging and suggested to us that Ba25 may be able to inhibit proliferation to alloantigens in a mixed lymphocyte reaction.

We looked at the potential role of Ba25 in modulating the functional activity of effector cells generated in a primary MLR. The first experiment investigated the effect of various concentrations of Ba25 on a 6 day primary mixed lymphocyte reaction.

2.3.1. Primary mixed lymphocyte reaction (MLR)

Ba25 dose dependently (12.5 – 200 nM) inhibited the primary mixed lymphocyte reaction (Figure 4). Two further dose response experiments confirmed that the relationship between the concentration of Ba25 and % suppression of the MLR was linear with logarithmic transformation ($p < 0.0001$) and the IC50 was ±135nM. A further set of 10 experiments with different donors was done, which confirmed the consistent inhibitory effect of a 30 min pre-incubation period of Ba25 (200 nM) on the MLR. For 10 experiments, the suppression ranged between 56.4% - 78% (mean 67.8%, median 66.5% suppression).

Figure 4. Effect of various concentrations of Ba25 on a 6 day primary mixed lymphocyte reaction



$1x10^5$responder cells (A) were incubated with $1x10^5$ irradiated (3000 R) stimulator cells (Bx) per well at 37°C in the absence or presence of Ba25 at varying concentrations (200, 100, 50, 25 and 12.5 nM).

Responder cells were pre-incubated with Ba25 for 30 min at 37ºC prior to the addition of stimulator cells.

On day 6, [^{3}H] thymidine was added, incorporation measured 18h later and results are expressed as mean DPM of 6 replicates ± SD.

[^{3}H] thymidine incorporation for A+Bx was 325 760 ± 15 774 DPM.

Results of a representative dose response are shown and are expressed as % suppression of proliferation ± SD based on the MLR (A+Bx) control. * $p < 0.0005$, ** $p < 0.014$, *** $p < 0.028$, **** $p < 0.004$, comparing % suppression at each successive concentration.

2.3.2. Lymphocyte populations and phenotype of the lymphocytes generated in a primary MLR in the absence or presence of Ba25

Flow cytometry was used to determine the phenotype of the effector cells generated in the primary MLR in the absence or presence of Ba25 (200 nM). Both the % of cells in the lymphocyte gate (set on forward and site scatter properties) expressing a particular receptor and the MFI (mean fluorescent intensity) of the antibody binding was determined. Lymphocytes in the lymphocyte gate were analysed for expression of activation markers HLA-DR and the IL-2 receptor (CD25) and NK cell markers in the form of CD16. Anti-CD3 was used to detect T cells. Anti-CD4 and anti-CD8 was used to determine T cell subsets.

Table 5. Lymphocyte populations and phenotype of effector cells generated in a 6 day primary MLR in the absence or presence of Ba25 (200 nM).

Receptor	% Positive cells		MFI		% Change	
	-Ba25	+Ba25 (200 nM)	-Ba25	+Ba25 (200 nM)	% Positive cells	MFI
Experiment 1 (A + Cx)						
CD3	91.3	86.4	8.4	6.1	No change	27.4% ↓
CD4	75.6	69.3	17.7	11.9	No change	32.8% ↓
CD8	31.4	34.0	4.1	4.5	No change	No change
CD16	11.0	10.3	4.6	4.1	No change	No change
HLA-DR	69.3	64.2	10.3	8.7	No change	No change
CD25	47.0	38.2	10.4	9.4	18.7% ↓	No change
CD4:CD8	2.4	2.0			16.7% ↓	
Total cell yield	$16.0x10^6$/ml	$6.8x10^6$/ml				
% suppression of proliferation		57.5% ↓				
Experiment 2 (B + Ax)						
CD3	89.4	90.1	7.8	8.8	No change	No change
CD4	67.7	67.5	14.5	10.3	No change	28.9% ↓
CD8	32.3	38.2	3.7	3.9	18.3% ↑	No change
CD16	6.5	6.1	3.1	2.8	No change	No change
HLA-DR	70.0	59.5	11.6	9.5	15.0% ↓	No change
CD25	53.1	48.7	12.6	11.6	No change	No change
CD4:CD8	2.1	1.8			14.3% ↓	
Total cell yield	$11.2x10^6$/ml	$5.7x10^6$/ml				
% suppression of proliferation		49.1% ↓				

Effector cells generated in 6 day bulk primary MLRs in the absence or presence of Ba25 (200 nM). PBMC were used from 3 different donors (A, B, C) in 4 experiments and were used as responder cells (A, B or C) or irradiated (3000 R) stimulator cells (Ax, Bx or Cx) as indicated. On day 6, the cells were harvested and the total cell yield determined by counting the number of PBMC x 10^6/ml proliferating in the MLR in the absence or presence of Ba25 (200 nM). Aliquots of cells were then labeled with antibodies directed to the indicated lymphocyte receptors and single colour analysis of antibody binding was done by flow cytometry on gated lymphocytes.

Data from the 4 experiments are shown and are the % of cells in the lymphocyte gate expressing the receptor and the MFI of the antibody binding. The change in these parameters in the presence of Ba25 is expressed as a % of that in the absence of Ba25. ↓ indicates a decrease from that in the absence of Ba25 and ↑ indicates an increase from that in the absence of Ba25.

A change in the % of cells expressing a specific receptor and a change in MFI in the presence of Ba25 was considered a real change if this was more than a 10% and 20% change respectively. Only real changes decided by these criteria are shown in the last 2 columns.

Table 5 cont. Lymphocyte populations and phenotype of effector cells generated in a 6 day primary MLR in the absence or presence of Ba25 (200 nM).

Receptor	% Positive cells		MFI		% Change	
	-Ba25	+Ba25 (200 nMm)	-Ba25	+Ba25 (200 nM)	% Positive cells	MFI
Experiment 3 (B + Cx)						
CD3	90.0	89.9	9.7	6.0	No change	38.1% ↓
CD4	64.6	55.0	13.3	8.4	14.9% ↓	36.8% ↓
CD8	37.3	46.3	3.9	4.1	24.1% ↑	No change
CD16	10.4	6.3	3.3	3.1	39.4% ↓	No change
HLA-DR	60.5	47.8	8.9	7.3	21.0% ↓	No change
CD25	48.7	40.7	11.2	10.1	16.4% ↓	No change
CD4:CD8	1.7	1.2			29.4% ↓	
Total cell yield	$8.4x10^6$/ml	$5.7x10^6$/ml				
% suppression of proliferation		32.1% ↓				
Experiment 4 (C + Bx)						
CD3	84.9	82.5	9.3	8.0	No change	No change
CD4	68.3	62.5	13.8	8.9	No change	35.5% ↓
CD8	33.9	36.4	3.7	4.0	No change	No change
CD16	14.3	11.0	3.1	2.9	23.1% ↓	No change
HLA-DR	62.4	44.8	13.2	11.1	28.2% ↓	No change
CD25	50.1	41.8	13.3	12.6	16.6% ↓	No change
CD4:CD8	2.0	1.7			15.0% ↓	
Total cell yield	$9.1x10^6$/ml	$2.3x10^6$/ml				
% suppression of proliferation		74.7% ↓				

Effector cells generated in 6 day bulk primary MLRs in the absence or presence of Ba25 (200 nM). PBMC were used from 3 different donors (A, B, C) in 4 experiments and were used as responder cells (A, B or C) or irradiated (3000 R) stimulator cells (Ax, Bx or Cx) as indicated. On day 6, the cells were harvested and the total cell yield determined by counting the number of PBMC x 10^6/ml proliferating in the MLR in the absence or presence of Ba25 (200 nM). Aliquots of cells were then labeled with antibodies directed to the indicated lymphocyte receptors and single colour analysis of antibody binding was done by flow cytometry on gated lymphocytes.

Data from the 4 experiments are shown and are the % of cells in the lymphocyte gate expressing the receptor and the MFI of the antibody binding. The change in these parameters in the presence of Ba25 is expressed as a % of that in the absence of Ba25. ↓ indicates a decrease from that in the absence of Ba25 and ↑ indicates an increase from that in the absence of Ba25.

A change in the % of cells expressing a specific receptor and a change in MFI in the presence of Ba25 was considered a real change if this was more than a 10% and 20% change respectively. Only real changes decided by these criteria are shown in the last 2 columns.

In summary, all 4 primary MLR (Table 5) confirmed that Ba25:

i) suppressed the mixed lymphocyte reaction as the total cell yield on day 6 was decreased (range of inhibition of total cell yield 32.1 – 74.7%, median 53.3%).

ii) had minimal effect on the generation of lymphocytes expressing the activation markers: HLA-DR (range of suppression 7.4 – 28.2%, median 18%); CD25 (range of suppression 8.3 – 18.7%, median 16.5%).

iii) had minimal effect on the MFI of HLA-DR expression (range of suppression 15.5 – 18.1%, median 16.9%) and had no effect on the MFI of CD25 expression (range of suppression 5.3 – 9.8%, median 8.8%).

iv) minimally impaired the generation of NK cells as determined by the number of cells expressing CD16 (range of suppression 6.2 – 39.4%, median 14.7%) and had no effect on the MFI of CD16 expression (range of suppression 6.1 – 10.9%, median 8.1%).

v) decreased the CD4:CD8 ratio (range of suppression 14.3 – 29.4%, median 15.9%).

2.4. Functional analysis of lymphocytes in the primary MLR

Lymphocytes from a primary MLR cultured in the presence of Ba25 were tested for suppressor cell activity on day 6 of the culture. Cells harvested from the primary MLR were incubated for 30 min, followed by 2 washes in fresh complete media to remove Ba25 before assessment of suppressor cell activity.

2.4.1. Suppressor cell activity of the primary MLR generated effectors on a fresh MLR

We investigated the ability of primed effector cells harvested from a bulk primary MLR in the presence of Ba25 to inhibit the proliferative response of a fresh MLR. We looked at both specific and non-specific suppression by primed effector cells generated in the presence of Ba25.

Table 6. Effect of Ba25 on the suppressor cell assays.

Response being tested in the fresh MLR	Primed cells from a bulk primary MLR added to a fresh MLR	Fresh MLR components	[^{3}H] Thymidine incorporation (DPM)	% Suppression of proliferation of fresh MLR
1. Autologous	(A + Bx)	A + Bx	60464 ± 1058	-
Autologous	(A + Bx+ 200nM Ba100)	A + Bx	27269 ± 969	54.9% ± 2.1
2. Heterologous	(A + Bx)	C + Ex	112428 ± 3542	-
Heterologous	(A + Bx+ 200nM Ba100)	C + Ex	60374 ± 1781	46.3% ± 1.2
3. Same stimulator, different responder	(A + Bx)	C + Bx	88532 ± 2128	-
Same stimulator, different responder	(A + Bx+ 200nM Ba100)	C + Bx	46745 ± 1062	47.2 % ± 1.8
4. Same responder, different stimulator	(A + Bx)	A + Cx	90058 ± 2117	-
Same responder, different stimulator	(A + Bx+ 200nM Ba100)	A + Cx	45506 ± 1120	49.4% ± 2.4

A bulk primary MLR was set up by incubating responder cells, A ($1x10^6$ cells/ml) with irradiated (3000 R) stimulator cells, Bx ($1x10^6$ cells/ml) in complete media in the absence or presence of Ba25 (200 nM) at 37°C. After 6 days incubation, the cells were harvested, incubated for 30 min, washed x 2 to remove Ba25 and used as the primed cells in the suppressor cell assay.

Fresh MLR reactions contained the component cells A+Bx, C+Ex, C+Bx, A+Cx: $1x10^5$ responder cells (A or C) were incubated with $1x10^5$ irradiated (3000 R) stimulator cells (Bx, Cx or Ex) per well in complete media at 37°C.

These fresh MLR (A+Bx, C+Ex, C+Bx and A+Cx) were cultured with the primed cells in the following combinations.
1. Primed cells autologous to responders and stimulators. 2. Primed cells, responders and stimulators completely heterologous. 3. Same stimulator, different responder. 4. Same responder, different stimulator.

On day 6, [^{3}H] thymidine was added and incorporation measured 18 h later.

Results of a representative experiment are shown and expressed as mean DPM of 6 replicates ± SD or % suppression of proliferation ± SD.

Effector cells generated in a primary MLR in the presence of Ba25 (200 nM) exerted a suppressive effect on the proliferation of a fresh MLR. This suppressor activity was not antigen specific (Table 6). This was reproducible in two further experiments.

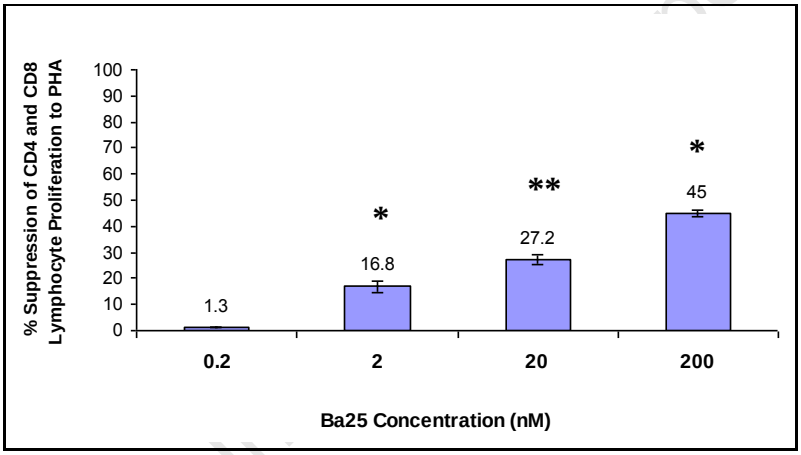
2.5. CD4 lymphocyte proliferation assays

We investigated the ability of Ba25 to inhibit isolated CD4 lymphocyte proliferation and whether Ba25 needed to be processed by the antigen-presenting phagocytes in order to exert its inhibitory effect on lymphocyte proliferation.

2.5.1. Effect of Ba25 on isolated CD4 and CD8 lymphocyte proliferation in response to PHA

We looked at the ability of Ba25 to inhibit CD4 and CD8 lymphocyte proliferation in response to PHA in the absence of antigen-presenting phagocytes. CD4 and CD8 lymphocytes were isolated using Dynal beads.

Figure 5. Effect of Ba25 on isolated CD4 and CD8 lymphocyte proliferation (60:40% CD4:CD8 population ratio) in response to PHA.



CD4 and CD8 lymphocytes were isolated using Dynal beads, then reconstituted in a 60:40% ratio (CD4:CD8). A total of $1x10^5$ CD4 + CD8 cells were cultured per well with PHA (0.00575 mitogenic units) or PHA + Ba25 (200, 20, 2 and 0.2 nM concentrations) at 37ºC.

Ba25 was pre-incubated with the CD4 and CD8 lymphocytes for 30 min at 37ºC prior to the addition of PHA.

At 72 h, [^{3}H] thymidine was added, incorporation measured 18 h later and results are expressed as mean DPM of 6 replicates ± SD.

Background counts in the absence of PHA or Ba25 were 220 ± 14 DPM.

[^{3}H] thymidine incorporation into PHA-stimulated CD4:CD8 lymphocytes was 15 666 ± 828 DPM.

Results of a representative dose response to Ba25 are shown and expressed as % suppression of PHA-induced CD4:CD8 lymphocyte proliferation ± SD based on the PHA-stimulated control. * $p < 0.002$, ** $p < 0.008$, comparing % suppression at each successive concentration.

Ba25 inhibited proliferation of isolated CD4 and CD8 lymphocytes (mixed at a ratio found in whole blood) in response to PHA (Figure 5). 3 dose response experiments confirmed that the relationship between the concentration of Ba100 and % suppression of CD4 and CD8 lymphocyte proliferation in response to PHA was linear with logarithmic transformation ($p < 0.0001$) and the IC50 was ± 343nM.

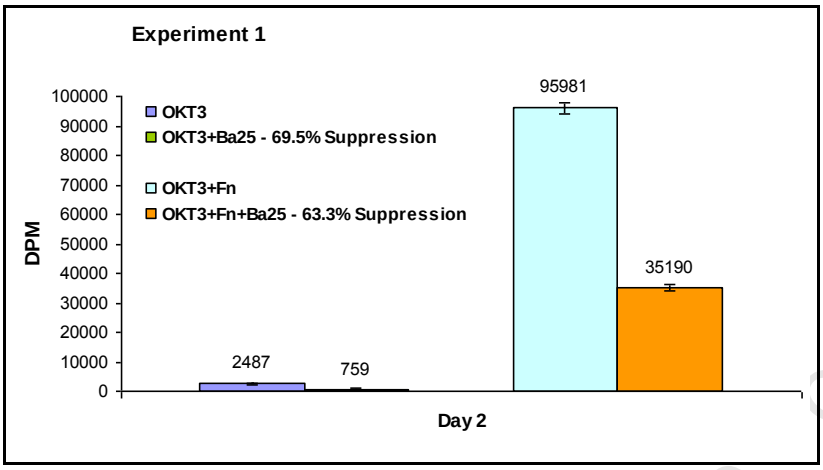
Ba25 at a concentration of 200 nM consistently inhibited proliferation of isolated CD4 and CD8 lymphocytes (mixed at a ratio found in whole blood) in response to PHA. The range of suppression for a further 5 experiments was 40-46.2% (mean 43.5%, median 44% suppression).

2.5.2. Effect of Ba25 on OKT3 and fibronectin-induced proliferation of CD4 lymphocytes

We next investigated the ability of Ba25 to modulate CD4 lymphocyte proliferation in response to OKT3 and fibronectin and thus its potential to interfere with cell-extracellular matrix interactions. The previous kinetic studies for Ba100 had shown that CD4 lymphocyte proliferation in response to OKT3 and fibronectin was maximal on day 2.

Figure 6a-e. Effect of Ba25 on OKT3 and fibronectin-induced proliferation of CD4 lymphocytes.

a.



b.

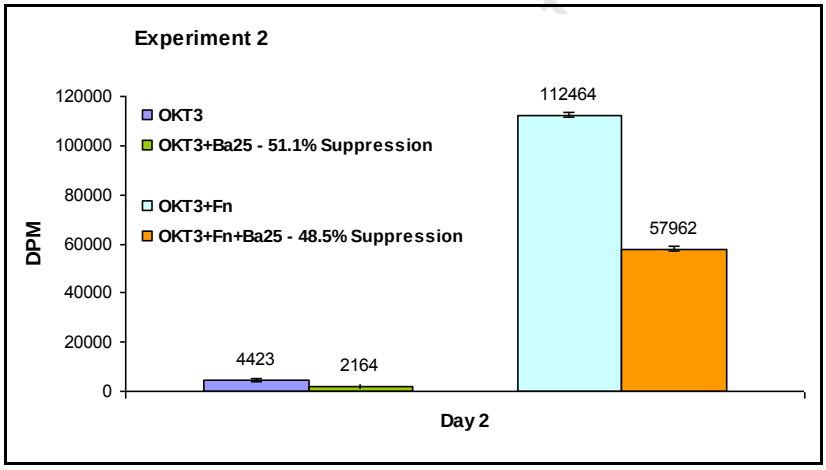
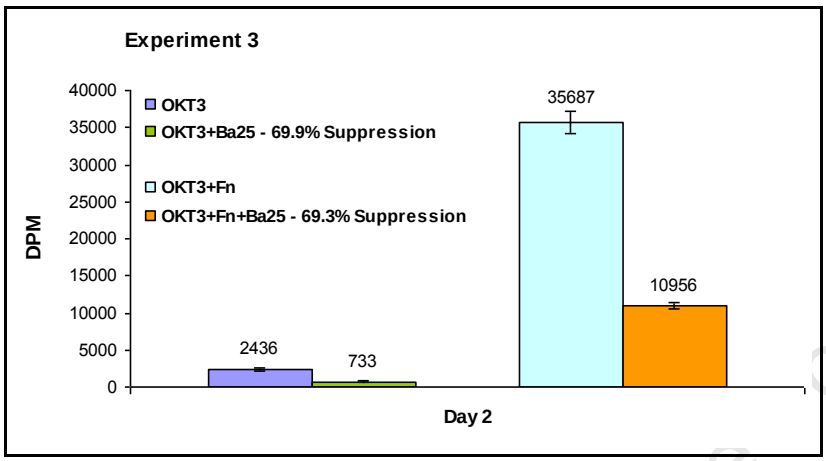


Figure 6a-e. Effect of Ba25 on OKT3 and fibronectin-induced proliferation of CD4 lymphocytes.

c.



d.

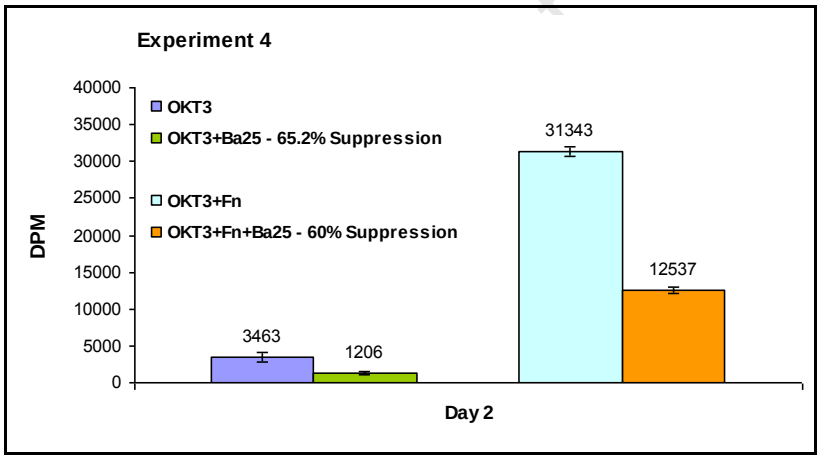
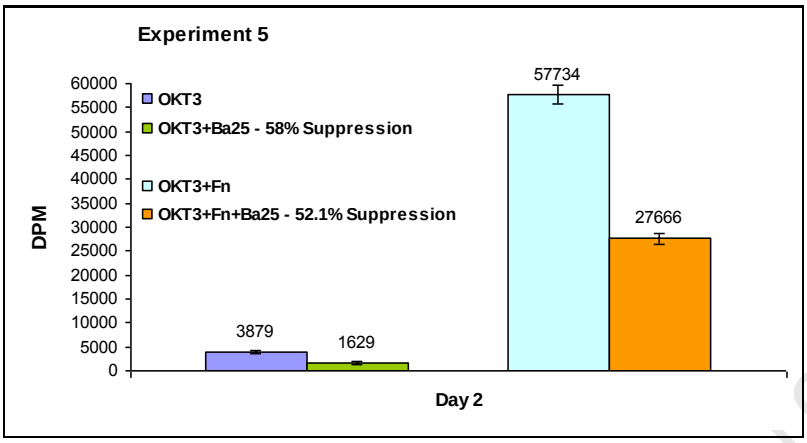


Figure 6a-e. Effect of Ba25 on OKT3 and fibronectin-induced proliferation of CD4 lymphocytes.

e.



CD4 lymphocytes were isolated from PBMCs using Dynal beads.

$1x10^5$ CD4 lymphocytes/well were incubated in OKT3-coated flat-bottomed wells in serum free culture medium (Serotec medium) with and without fibronectin (30 µg/ml) in the absence or presence of Ba25 (200 nM) at 37°C.

Ba25 was pre-incubated with CD4 lymphocytes for 30 min at 37°C before the addition of fibronectin to the wells.

[^{3}H] thymidine was added on day 2 and incorporation measured 18 h later.

Background [^{3}H] thymidine incorporation for cells only and cells + Ba25 or + fibronectin alone never averaged above 247 DPM.

Results are expressed as mean DPM of 6 replicates ± SD.

Ba25 at a concentration of 200 nM consistently inhibited CD4 lymphocyte proliferation (2 day proliferation assay) in response to OKT3 and OKT3 with fibronectin (Figure 6a-e). The range of suppression of:

i) OKT3-induced CD4 lymphocyte proliferation was 51.1–69.9% (mean 62.7%, median 65.2% suppression).

ii) OKT3 and fibronectin-induced CD4 lymphocyte proliferation was 48.5–69.3% (mean 58.6%, median 60.0% suppression).

2.6. Effect of Ba25 on PBMC proliferation in response to anti-CD28 antibody and a phorbol ester, PMA

We found that Ba25 inhibited calcium-dependent T lymphocyte proliferation pathways involving TCR/CD3 interactions with mitogen, antigen and allo-antigen, as well as T cell receptor/integrin receptor stimulated proliferation.

We then investigated whether Ba25 could inhibit calcium-independent proliferation pathways such as proliferation in response to anti-CD28 antibody and a phorbol ester, PMA.

Table 7. Effect of Ba25 on PBMC proliferation in response to anti-CD28 antibody and PMA.

	[^{3}H] Thymidine incorporation (DPM)	Effect on PBMC proliferation
PMA	22 220 ± 165	-
PMA + anti-CD28 antibody	64 855 ± 2543	-
PMA + anti-CD28 antibody + Ba25 (200 nM)	58 629 ± 2041	9.6% ± 1.06

PBMC ($0.5x10^6$ cells/well) were incubated in 1% BSA/PBS with PMA (1 ng/ml); PMA + anti-CD28 antibody (1 ng/ml) or PMA + anti-CD28 antibody + Ba25 (200 nM) at 37°C.

Ba25 was pre-incubated with PBMC for 30 minutes at 37°C prior to the addition of PMA or PMA + anti-CD28.

At 72 h, [^{3}H] thymidine was added and incorporation measured 18 h later.

Results of a representative experiment are shown and expressed as mean DPM of 6 replicates ± SD or % suppression of PBMC proliferation to anti-CD28 antibody/PMA ± SD.

Ba25 at a concentration of 200 nM was unable to inhibit PBMC proliferation in response to anti-CD28 antibody and PMA (Table 7). This was reproducible in 4 experiments.

2.7. Effect of Ba25 on processes involved in T lymphocyte proliferation

Our data indicated that Ba25 inhibited calcium-dependent, but not calcium-independent T lymphocyte proliferation pathways. In addition, Ba25 must be added within 2 hours of initiation of lymphocyte proliferation in order to exert its inhibitory effect, suggesting that Ba25 acts early in the T cell activation pathway leading to proliferation.

To obtain further insight into the action of Ba25, we investigated the effect of Ba25 on:

i) Cytokine production during a mixed lymphocyte reaction.

ii) The ability of exogenous IL-2 to reverse the inhibition of PHA-induced PBMC proliferation in the presence of Ba25.

iii) Cell cycle progression during PHA-induced PBMC proliferation.

iv) Proliferating cell nuclear antigen expression.

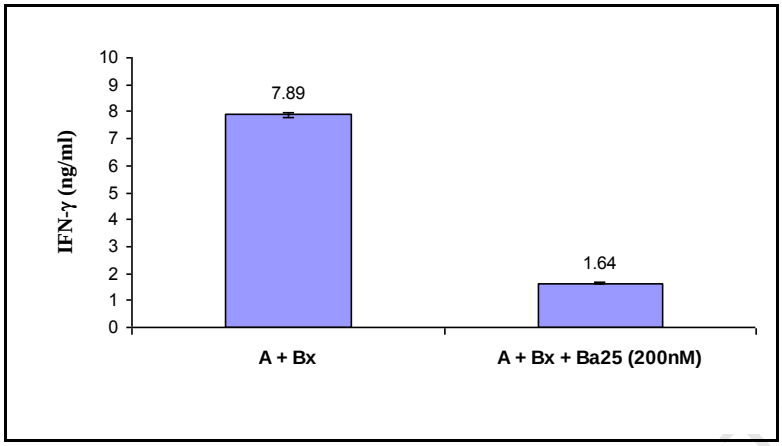
v) Lymphocyte membrane stability.

2.7.1. Effect of Ba25 on IFN-γ, IL-4 and IL-10 production during the mixed lymphocyte reaction

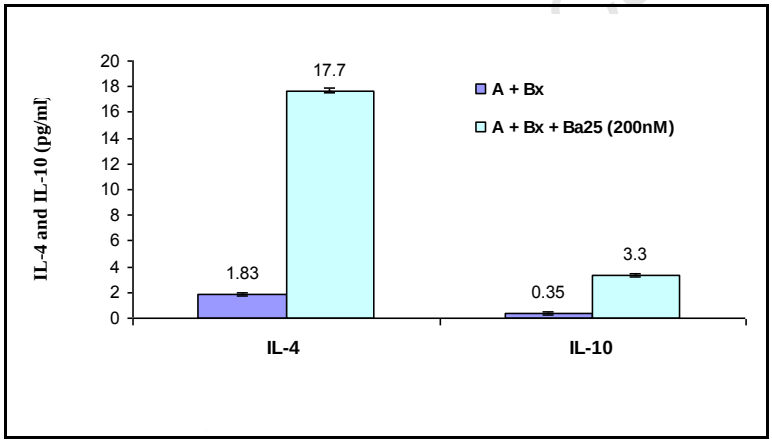
We have shown that Ba25 inhibits T lymphocyte proliferation, but had little effect on the generation of lymphocytes expressing the activation markers HLA-DR and CD25 and did not alter lymphocyte IL-2 receptor expression. We next investigated whether Ba25 altered cytokine production during a mixed lymphocyte reaction.

Figure 7a-b. Effect of Ba25 on cytokine production during a mixed lymphocyte reaction

a.



b.



$1x10^5$ responder cells (A) were incubated with $1x10^5$ irradiated (3000R) stimulator cells (Bx) per well at 37°C in the absence or presence of Ba25 (200 nM).

Responder cells were pre-incubated with Ba25 for 30 min at 37°C prior to the addition of stimulator cells.

On day 6, [^{3}H] thymidine was added and incorporation measured 18 h later to confirm the inhibitory effect of Ba25 on the MLR or cells were harvested and the supernatant collected for cytokine analysis by ELISA.

Cytokine production in the presence of A+Bx with and without Ba25 was within the sensitivity range of the ELISA.

Results of two representative experiments are shown.

Ba25 inhibited the MLR (52% inhibition), induced a Th2 cytokine response (increased production of IL-4 and IL-10) and inhibited the production of the Th1 cytokine IFN-γ (Figure 7a-b). This was confirmed in two further experiments.

Ba25 at a concentration of 200 nM induced a Th2 cytokine profile.

i) Inhibited IFN-γ production: 2.2 – 4.8 fold decrease, median 3.6 fold decrease.

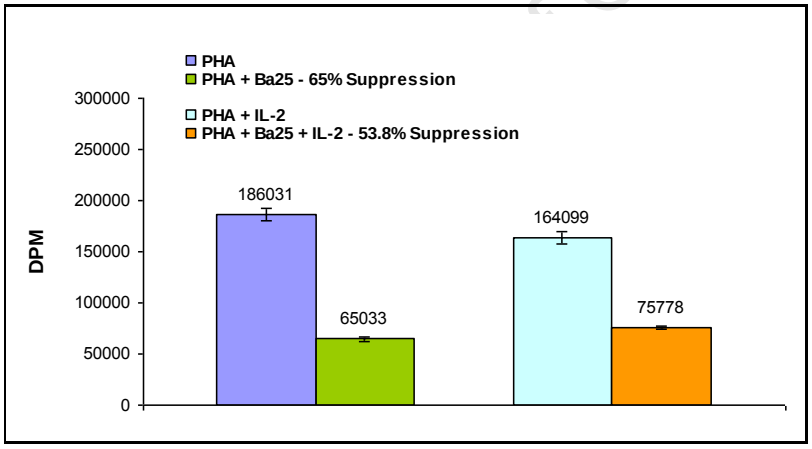
ii) Stimulated IL-4 production: 8.3 – 27.5 fold increase, median 9.7 fold increase.

iii) Stimulated IL-10 production: 6.7 – 20.7 fold increase, median 9.4 fold increase.

2.7.2. Effect of addition of exogenous IL-2 on PHA-induced PBMC proliferation in the presence of Ba25

We then looked at whether the addition of exogenous IL-2 could reverse the inhibitory action of Ba25 on PHA-stimulated lymphocytes or whether other pathways besides cytokines were involved in the immunomodulation.

Figure 8. Effect of addition of exogenous IL-2 on PHA-induced PBMC proliferation in the presence of Ba25.



PBMC (1x10^5 cells/well) were incubated with PHA (0.00575 mitogenic units); PHA + Ba25 (200 nM); PHA + IL-2 (10 U/ml) and PHA + Ba25 + IL-2 at 37ºC.

Ba25 was pre-incubated with PBMC for 30 min at 37ºC prior to the addition of PHA or PHA + IL-2.

At 72 h, [^{3}H] thymidine was added and incorporation was measured 18 h later.

Background [^{3}H] thymidine incorporation into PBMC in the absence of PHA, Ba25 or IL-2 was 388 ± 24 DPM.

Results of a representative experiment are shown and expressed as the mean DPM of 6 replicates ± SD or as % suppression of PBMC proliferation ± SD.

In the continual presence of Ba25, the addition of exogenous IL-2 was unable to reverse the inhibitory effect of Ba25 (200 nM) on PHA-induced lymphocyte proliferation (Figure 8). This was confirmed in 2 further experiments ($p = 0.1$ using Mann-Whitney Non-parametric test). This suggested that Ba25 interferes with other pathways, besides IL-2, involved in mitogen-induced T lymphocyte proliferation. Ba25 had been shown to induce a Th2 cytokine profile.

2.7.3. Effect of Ba25 on the distribution of PHA-stimulated PBMCs in the various phases of the cell cycle

We next investigated the effect of Ba25 on cell cycle progression.

The percentage of cells in the G_0G_1, S and G_2M phases during stimulation with PHA were measured at 24 and 48 h. At 24 h, most of the cells were in the G_0G_1 phase with minimal progression into the S and G_2M phases. At 48 h, cell cycling had progressed with cells now being detectable in the S and G_2M phases.

Ba25 (200 nM) had minimal effect on cell cycle progression in response to PHA stimulation (Table 8 and Figure 9a and b).

Table 8. Effect of Ba25 on the distribution of PHA-stimulated PBMC in the various phases of the cell cycle (24 and 48 h stimulation).

Stimulation	Phase	% Cells per cell cycle phase	
		- Ba25	+ Ba25 (200 nM)
Experiment 1. 24 h	G_0G_1	90	90
	S	8	8
	G_2M	2	2
Experiment 2. 48 h	G_0G_1	60	67
	S	20	17
	G_2M	16	14
Experiment 3. 48 h	G_0G_1	58	64
	S	22	20
	G_2M	17	15
Experiment 4. 48 h	G_0G_1	59	66
	S	21	19
	G_2M	18	16

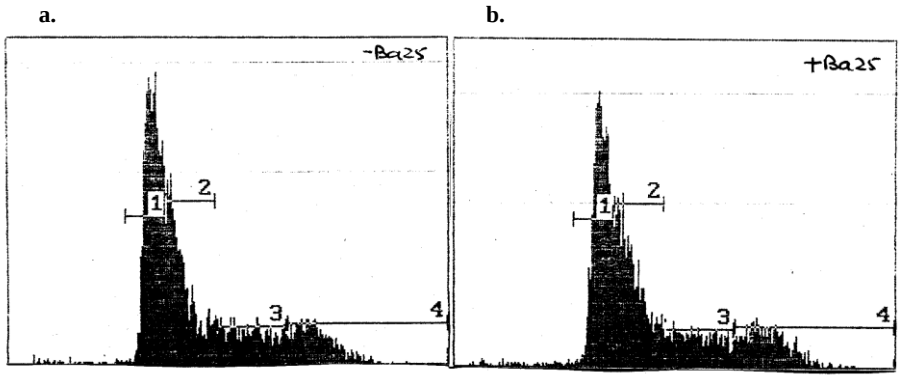
PBMC ($1x10^6$ cells/ml RPMI 1640/10% AB serum) stimulated with PHA in 30 ml tissue culture flasks in the absence or presence of Ba25 (200 nM) were harvested at 24 and 48 h and resuspended at $5x10^6$ cells/ml RPMI 1640/10% AB serum.

Cells were stained with propidium iodide (PI) for DNA quantitation by FACS.

Cells were permeabilised with non-ionic detergent and treated with PI and RNAse.

Single parameter histograms using FL detector 3 (emission spectrum peaks at 650 nm) were used to assess results.

Figure 9a and b. Effect of Ba25 on the distribution of PHA-stimulated PBMC in the various phases of the cell cycle at 48 h.



Phases of the cell cycle at 48 h: G_0G_1 phase (1and 2), S phase (3) and G_2M phase (4) in the absence of Ba25 (Fig. 9a) and in the presence of Ba25 (Fig. 9b).

2.7.4. Effect of Ba25 on proliferating cell nuclear antigen (PCNA) expression by PHA-stimulated PBMCs

We then investigated the effect of Ba25 on PCNA expression.

Table 9. Effect of Ba25 on proliferating cell nuclear antigen (PCNA) expression by PHA-stimulated PBMC (48 h stimulation).

Experiment	- Ba25		+ Ba25 (200 nM)		% Change	
	% Positive cells	MFI	% Positive cells	MFI	% Positive cells	MFI
1	72	2.5	68	2.4	5.6% ↓	4.0% ↓
2	64	1.7	65	1.8	1.6% ↑	5.9% ↑
3	39	1.9	39	1.9	0%	0%

PBMC ($1x10^6$ cells/ml RPMI 1640/10% AB serum) stimulated with PHA in 30 ml tissue culture flasks in the absence or presence of Ba25 (200 nM) were harvested at 48 h and resuspended at $2x10^6$ cells/ml RPMI 1640/10% AB serum.

Cells were permeabilised with 20 µg/ml lysolecithin, fixed with 1% paraformaldehyde for 2 min and then treated on ice for 10 min with methanol, followed by incubation on ice with 0.1% NP-40 for 5 min.

The intracellular nuclei were then stained with anti-PCNA and analysed by FACS.

Results are expressed as % cells expressing PCNA or as MFI which represents the extent of PCNA expression per cell. The change in PCNA expression in the presence of Ba25 is expressed as a % of that in the absence of Ba25. ↓ indicates a decrease from that in the absence of Ba25 and ↑ indicates an increase from that in the absence of Ba25.

Ba25 at a concentration of 200 nM did not inhibit the generation of cells expressing PCNA nor did it downregulate lymphocyte PCNA expression (Table 9).

2.7.5. Effect of Ba25 on lymphocyte membrane stability

Ba25 inhibited T lymphocyte proliferation and induced a Th2 cytokine profile. Ba25 had minimal effect on the generation of lymphocytes expressing the activation markers HLA-DR and CD25 and did not affect cell cycle progression nor PCNA expression. We then investigated whether the immunomodulatory effects of Ba25 might be due to effects on lymphocyte membrane stability and the catabolism of the major plasma membrane phospholipid, phosphatidylcholine with production of immunosuppressive metabolites.

We investigated whether Ba25 exerted its immunomodulatory effects via the phospholipase breakdown products of phosphatidylcholine. The major degradation products of phospholipase A_2 are lysophosphatidylcholine and arachidonic acid. Lysophosphatidylcholine and prostaglandin E2 (arachidonic acid metabolite) are inhibitory to lymphocyte proliferation.

i) Effect of α-tocopherol on PHA-induced PBMC proliferation in the presence of Ba25

α-Tocopherol stabilizes membrane phospholipids, preventing degradation by phospholipase A_2 by forming complexes with the lysophospholipids (Kagan 1989; Anderson et al 1993).

We investigated whether α-tocopherol was able to reverse the inhibitory effects of Ba25 on PHA-induced PBMC proliferation.

α-Tocopherol at a concentration of 50 µg/ml and 100 µg/ml was unable to reverse the inhibitory effects of Ba25 on PHA-induced PBMC proliferation. This was reproducible in 3 further experiments (Table 10).

Table 10. Effect of α-tocopherol on PHA-induced PBMC proliferation in the presence of Ba25.

Reaction	[^{3}H] Thymidine incorporation (DPM)	Suppression of PBMC proliferation
Cells only	445 ± 47	-
PHA	817 310 ± 1320	-
PHA + Ba25 (200 nM)	470 381 ± 1090	42.4% ±1.97
PHA + α-tocopherol (50 μg/ml) + Ba25 (200 nM)	482 213 ± 1954	41.0% ± 1.8
PHA α-tocopherol (100 μg/ml) + Ba25 (200 nM)	506 577 ± 1281	38% ± 2.26

PBMC (1x10^5 cells/well) were incubated with PHA (0.00575 mitogenic units); PHA + Ba25 (200 nM); α-tocopherol (50 μg/ml or 100 μg/ml) + Ba25 (200 nM) + PHA at 37°C.

PBMC were pre-incubated with Ba25 (200 nM) or Ba25 + α-tocopherol for 30 min at 37°C prior to the addition of PHA.

At 72 h, [^{3}H] thymidine was added and incorporation measured 18 h later.

Results of a representative experiment are shown and are expressed as the mean DPM of 6 replicates ± SD or as % suppression of PBMC proliferation to PHA ± SD.

ii) Effect of Indomethacin on PHA-induced PBMC proliferation in the presence of Ba25

Indomethacin, a non-selective cyclooxygenase inhibitor, was able to reverse the inhibitory effect of Ba25 on PHA-induced PBMC proliferation. This was reproducible in 3 further experiments (Table 11).

Table 11. Effect of Indomethacin on PHA-induced PBMC proliferation in the presence of Ba25.

Reaction	[^{3}H] Thymidine incorporation (DPM)	Suppression of PBMC proliferation
Cells only	396 ± 18	-
PHA	663 246 ± 1682	-
PHA + Ba25 (200 nM)	336 666 ± 2142	49.2% ± 2.47
PHA + Indomethacin (10 μg/ml) + Ba25 (200 nM)	617 413 ± 2846	6.9% ± 0.42
PHA + Indomethacin (5 μg/ml) + Ba25 (200 nM)	627 477 ± 2348	5.4% ± 0.56

PBMC (1x10^{5}cells/well) were incubated with PHA (0.00575 mitogenic units); PHA + Ba25 (200nM); indomethacin (10 μg/ml or 5 μg/ml) + Ba25 (200 nM) + PHA at 37°c.

PBMC were pre-incubated with Ba25 (200 nM) or Ba25+ Indomethacin for 30 min at 37°C prior to the addition of PHA.

At 72 h, [^{3}H] thymidine was added and incorporation measured 18 h later.

Results of a representative experiment are shown and are expressed as the mean DPM of 6 replicates ± SD or as % suppression of PBMC proliferation to PHA ± SD.

3. DISCUSSION

Ba25, a novel C-type lectin has been isolated from the venom of the Western Cape, South African puff adder, *Bitis arietans*. Functional studies demonstrated the ability of Ba25 to cause agglutination of platelets in platelet-rich plasma without the degranulation of platelets. Ba25 interacts with the GPIbα receptor of the von Willebrand receptor complex, GPIb-IX-V complex as well as the collagen receptor $\alpha_2\beta_1$ leading to activation of the platelet fibrinogen receptor GPIIbIIIa, increased fibrinogen binding to the GPIIbIIIa receptor and consequent platelet agglutination. Ba25 induces platelet agglutination without associated platelet activation (Jennings et al 2005, Jennings unpublished data). The thromboelastogram studies looking at the effect of Ba25 on whole blood clotting, especially the reduction in the reaction time, suggested that Ba25 was an inhibitor of early stage blood clotting events such as cell-cell or cell-surface interactions and had no direct effect on fibrinogen (Jennings et al 2005). Thus, Ba25 modulates platelet-platelet interactions and coagulation processes involved in thrombus formation.

Platelets play an important role in inflammation including the alloresponse leading to allograft injury (Zhang et al 2003, 2005; Xu et al 2006). Activated platelets promote activated T cell adhesion to fibronectin via integrin ($\alpha_5\beta_1$ and $\alpha_L\beta_2$), CD40-CD40 ligand and P-selectin-PSGL-1 mediated interactions (Shenkman et al 2006). The degree of platelet activation pre-transplantation (increased surface expression of CD61, CD63 and PAC-1) has been shown to clinically correlate with acute rejection (Zhang et al 2003).

Platelets and lymphocytes have a number of receptors in common including the collagen receptor $\alpha_2\beta_1$ (Pribila et al 2004, Kasirer-Friede et al 2007). We therefore postulated that Ba25 with its ability to modulate platelet interactions by targeting receptors without associated activation and to prevent blood clotting by interfering with cellular interactions, might be able to modulate lymphocyte function and inhibit important processes involved in the alloimmune response. We therefore investigated the ability of Ba25 to modulate T lymphocyte proliferative processes.

Ba25 consistently inhibited in a dose dependent manner lymphocyte proliferation in response to mitogen (IC50 ± 138 nM), antigen (IC50 ± 20-118 nM depending on the antigen) and alloantigen (IC50 ± 135 nM) stimulation. Curve estimation (SPSS) was used to analyse the relationship between the concentration of Ba25 and the % suppression of lymphocyte proliferation in response to mitogen, antigens and alloantigens. The concentration curves for the mitogen PHA, the antigens (PPD, Tetanus toxoid and Varidase) and the MLR were linear with logarithmic transformation.

Ba25 was not lymphotoxic as the PBMCs remained viable despite prolonged culture in the presence of Ba25. Viability was confirmed microscopically at the end of each study by trypan blue exclusion or by exclusion of propidium iodide where the effect of Ba25 on the cell cycle in response to PHA was investigated. Preincubation with Ba25 was not required for its anti-proliferative effects as the suppression of proliferation was the same with no pre-incubation, 30 minutes or 60 minutes pre-incubation. If Ba25 was washed away after a 30 minute pre-incubation period prior to the addition of PHA, the inhibitory effect of Ba25 was lost. These results suggested that Ba25 did not interfere with any early plasma membrane associated events in T cell activation.For standardization purposes, a 30 minute pre-incubation period was used in all the experiments. The inhibitory effect of Ba25 on PHA-induced PBMC proliferation was not achieved, if Ba25 was added 2 hours after the initiation of PBMC proliferation. This suggested that Ba25 interferes with early events of T cell activation prior to DNA synthesis and cell division.

The addition of exogenous IL-2 did not reverse the inhibitory effects of Ba25 on PHA-induced PBMC proliferation, suggesting that Ba25 interferes with mitogen-induced T cell proliferation at more than one site.

Having established that Ba25 did possess immunosuppressive properties with respect to mitogen and antigens, we next looked at the *in vitro* model of transplantation, the primary mixed lymphocyte reaction. Ba25 inhibited the primary mixed lymphocyte reaction in a dose dependent manner. Effector cells generated in a primary MLR in the presence of Ba25 also exerted a suppressive effect on the proliferation of a fresh MLR. This suppressive activity was not antigen specific.

After establishing the inhibitory effects of Ba25 on lymphocyte proliferation, we used flow cytometry to look at the effect of Ba25 on lymphocyte activation markers and T cell subsets. Phenotypic analysis of lymphocytes after PHA-induced PBMC proliferation in the presence of Ba25 consistently showed that Ba25:

i) had minimal effect on both the generation of activated lymphocytes expressing HLA-DR and the number of HLA-DR receptors expressed per cell.

ii) had no effect on the generation of activated lymphocytes expressing CD25 (the IL-2 receptor) and did not affect cellular expression of the IL-2 receptor.

iii) minimally impaired the generation of NK cells expressing CD56 (median 22.8% suppression).

iv) decreased the CD4:CD8 ratio possibly due to decreased proliferation of CD4 lymphocytes.

Phenotypic analysis of Ba25-treated effector cells generated in a 6 day primary MLR confirmed that Ba25 had minimal effect on the generation of activated lymphocytes expressing the activation markers HLA-DR and CD25. Ba25 had minimal effect on the number of HLA-DR receptors expressed per cell and did not affect the cellular expression of CD25. Ba25 decreased the CD4:CD8 ratio.

Thus, the immunomodulatory effects of Ba25 did not appear to be due to inhibition of the generation of activated T lymphocytes, but rather a marked reduction in overall proliferation. Minimal suppression of NK cells was observed. The decreased CD4:CD8 ratio in response to PHA and alloantigen stimulation suggested that the proliferation of CD4 lymphocytes was being suppressed by Ba25. We next looked at the effect of Ba25 on CD4 lymphocytes which are pivotal to the alloimmune response.

Ba25 consistently inhibited isolated CD4 and CD8 lymphocyte proliferation in response to PHA in the absence of antigen-presenting phagocytes. This suggested that no processing of Ba25 by antigen-presenting phagocytes was required for its immunomodulatory activity. Furthermore, Ba25 inhibited CD4 lymphocyte proliferation in response to OKT3 and OKT3 and fibronectin. As fibronectin plays an

important role in the effector phase of the alloimmune response promoting adhesion and migration of alloreactive cells into the allograft (Coito et al 1998; Korom et al 1998; Coito et al 2000a,b), this inhibitory effect of Ba25 is potentially beneficial.

Ba25 inhibited calcium-dependent T lymphocyte proliferation pathways, but was unable to inhibit calcium-independent T lymphocyte proliferation pathways (PBMC proliferation in response to anti-CD28 antibody and PMA).

Having demonstrated that Ba25 inhibited CD4 lymphocyte proliferation with minimal modulation of the activation markers (HLA-DR and CD25), we investigated whether Ba25 could be inducing an immunosuppressive cytokine profile. Ba25 was consistently shown to induce a Th2 cytokine response increasing IL-4 and IL-10 and decreasing IFN-γ production. IL-10 is a potent anti-inflammatory and immunosuppressive cytokine (Zhou et al 2005; Taylor et al 2006). IL-10 inhibits CD28 tyrosine phosphorylation, preventing the binding of phosphatidylinositol 3-kinase p85, thereby inhibiting the CD28 signaling pathway (Taylor et al 2006). IL-10 has been shown to impair the DTH response (VanBuskirk et al 2000), suppressing the alloimmune response and to be a marker of immune tolerance in animal models of transplantation (Blancho et al 1995; Arai et al 1999; Shinozaki et al 1999; Tashiro et al 2000). Thus, IL-10 is an important regulator of the alloimmune response.

IL-4 is a pleiotropic cytokine which promotes the differentiation of Th2 effectors, and inhibits the development of Th1 responses (Le Gros et al 1990; Swain et al 1990; Skapenko et al 2004). IL-4 also helps to maintain optimal regulatory activity of $CD4^+$ $CD25^+$ regulatory T cells (Yates et al 2007).

A Th2 alloimmune response (IL-4 and IL-10) is able to regulate indirect Th1 alloimmune responses, preventing chronic rejection and promoting tolerance (Kist-van Holthe 2002). IL-4 and IL-10 also play a regulatory feedback role by inhibiting COX-2 gene expression and PGE_2 production in mature dendritic cells (Teloni et al 2006), neutrophils (Niiro et al 1997) and monocytes (Niiro et al 1998).

Ba25 caused minimal change in the number of lymphocytes cycling in response to PHA stimulation and did not alter PCNA expression. We next investigated whether Ba25 affected lymphocyte membrane stability and the production of immuno-inhibitory prostaglandins. α-Tocopherol which stabilizes membrane phospholipids by forming

complexes with lysophospholipids and preventing degradation by phospholipase A_2, was unable to reverse the inhibitory effects of Ba25. Indomethacin, a non-selective cyclooxygenase inhibitor was able to reverse the inhibitory effects of Ba25 on PHA-induced PBMC proliferation. This suggested that Ba25 might be inducing the production of the immuno-inhibitory prostaglandin E_2 through the activation of cyclooxygenase.

Phosphatidylcholine is the major phospholipid in plasma membranes and is catabolised through the hydrolytic action of phospholipase A1 or A2. The major degradation products of phosphatidylcholine are lysophosphatidylcholine and arachidonic acid. Arachidonic acid can be metabolised to a number of immunologically active products including prostanoids (prostaglandins and thromboxane), leukotrienes, isoprostones and p450 metabolites (HETE). The prostanoids and leukotrienes have been shown to play a role in the alloimmune response with thromboxane and leukotrienes promoting allograft injury and prostaglandin E_2 promoting allograft survival (Tilley et al 2001; Rocha & Carvalho 2005).

Arachidonic acid is converted to prostaglandin H2 by the cyclooxygenase enzymes COX-1 and COX-2 (Vane et al 1998). PGH2 is then acted on by prostaglandin synthases to produce the various inhibitory prostaglandins including PGI_2 and PGE_2 (Kingston et al 1985). Arachidonic acid may also be converted by thromboxane synthase to thromboxane and hydroxylicosate traenoic acid (HETE) which stimulate lymphocyte responses to mitogen (Kelly et al 1979; Tilley et al 2001). Animal models of allograft rejection have shown that thromboxane impairs graft function (Tannenbaum et al 1984; Coffmann et al 1985; Mangino et al 1987; Thomas et al 2003). In contrast, prostaglandin E_2 has been shown to prolong survival of renal (Strom & Carpenter 1983), cardiac (Fabrega et al 1992) and small intestine (Koh et al 1992) allografts in rats.

PGE_2 modulates macrophage function, downregulating the expression of MHC class II antigens (Snyder et al 1982), inhibiting IL-1 (Kunkel et al 1986), IL-12 (van der Pouw Kraan 1995, 1996), TNF-α (Scales et al 1989) and superoxide (Metzger et al 1981) production and increasing IL-10 production in response to LPS (Harizi et al 2002).

PGE_2 has diverse effects on the regulation and activity of T cells, predominantly CD4 lymphocytes. PGE_2-induced inhibition of T lymphocyte proliferation involves inhibition of polyamine synthesis (Ruggeri et al 2000), inhibition of intracellular calcium release (Choudhry et al 1999a) and suppression of p59 (Fyn) protein tyrosine kinase activity (Choudhry et al 1999b). PGE_2 binds to G-protein complex receptors E2 and E4 and suppresses T cell immune responses by eliciting a cAMP (protein kinase A) Csk inhibitory pathway localized to the lipid rafts (Vang et al 2001, 2003; Okano et al 2006).

PGE_2 decreases chemokine production in various cell types. It inhibits CCL3 and CCL4 production in dendritic cells (Jing et al 2003) and inhibits CCL27 production in keratinocytes (Kanda et al 2004). PGE_2 is in important modulator of DC function including its cytokine production, increasing IL-10 production and downregulating IL-12 production (Harizi et al 2002). PGE_2 has been shown to promote polarization of dendritic cells into mature cells that promote naïve T cell differentiation into Th2 cells. PGE_2 upregulates DC production of Th2-attracting chemokines CCL17 and CCL22 and downregulates IFN-γ induced CXCL10 production by immature DC (McIlroy et al 2006). Dendritic cells have also been shown to expand antigen-specific $FOXP3^+$ $CD25^+$ $CD4^+$ Tregs (Yamakazi et al 2006).

PGE_2 differentially affects apoptosis in resting mature T cells and activated T cells. PGE_2 induces apoptosis of resting mature T cells via a c-Myc dependent and Bcl-2 independent pathway (Pica et al 1996). PGE_2 protects T cells from TCR-mediated activation induced cell death by decreasing T cell Fas-ligand expression (Porter et al 1999).

PGE_2 has been shown to induce a Th2 cytokine response, enhancing IL-4 and IL-10 production and inhibiting production of Th1 cytokines, IFN-γ and IL-2 by Th1 cells (Snijdewint et al 1993; Katamura et al 1995; Hilkens et al 1996; Huang et al 1998; Stolina et al 2000; Pockaj et al 2004). PGE_2 inhibits CD8 lymphocyte proliferation and decreases CD8 lymphocyte IFN-γ production (Hendricks et al 2000; Ganapathy et al 2000).

PGE_2 inhibits the human MLR, inhibiting the upregulation of ICAM-1, B7-1 (CD80) and B7-2 (CD86) on monocytes and inhibits IL-12 and IFN-γ production. This occurs through the stimulation of the EP2 and EP4 receptors leading to cAMP production (Morichika et al 2003).

PGE_2 induces the expression of functional inhibitory CD94/NKG2A receptors (a C-type lectin receptor) on $CD8^+$ T lymphocytes via a cAMP-dependent protein kinase A type I pathway (Zeddou et al 2005).

In addition to these effects on CD4 and CD8 lymphocytes, PGE_2 also exerts effects on $CD4^+$ $CD25^+$ Treg cells, which play a role in maintaining peripheral immune tolerance and inhibiting autoreactive T cells (Powrie et al 1994; Sakaguchi 2000; Shevach 2002). Treg cells have been shown to effectively inhibit effector T cell responses such as cytokine production and proliferation (Thornton & Shevach 1998). Treg cells have increasingly been shown to play a role in longterm allograft acceptance and transplantation tolerance (Jiang et al 2006a; Yong et al 2007; Kang et al 2007; Bestard et al 2007; Kitazawa et al 2007; Coenen et al 2007b).

Prostaglandin E2 has been shown to enhance the *in vitro* inhibitory function of human purified $CD4^+$ $CD25^+$ Treg cells and is also able to induce a regulatory phenotype in $CD4^+$ $CD25^-$ T cells (Baratelli et al 2005). PGE_2 induces the Treg cell-specific transcription factor FOXP3 (Forkhead/winged helix transcription factor) gene in $CD4^+$ $CD25^-$ T cells and upregulates its expression in $CD4^+$ $CD25^+$ Treg cells. PGE_2 upregulates FOXP3 at both mRNA and protein levels and enhances FOXP3 promotor activity (Baratelli et al 2005).

IL-2 is unable to reverse PGE_2-induced or $CD4^+$ $CD25^+$ Treg cell-induced suppression (Baratelli et al 2005). PGE_2 induces FOXP3 expression in a cAMP dependent manner in both $CD4^+$ $CD25^-$ and $CD4^+$ $CD25^+$ T cells. The $CD4^+$ $CD25^+$ adaptive Treg cells express COX-2 and suppress effector T cells via a PGE_2-dependent mechanism (Mahic et al 2006).

Overexpression of cyclooxygenase-2 and prostaglandin E2 secretion in breast cancer patients has been shown to be associated with reduced T cell effector function, decreased dendritic phagocytic cell function and a Th2 cytokine profile (increased IL-4 and IL-10, decreased IFN-γ, TNF-α, IL-12 and IL-2) (Pockaj et al 2004).

Thus, the immunosuppressive effects of Ba25 may be due to a combination of the direct inhibitory effects of prostaglandin E2 on T lymphocyte proliferation, the ability of PGE_2 to promote a Th2 cytokine profile and to modulate Treg function. The status of Treg cells during proliferation in the presence of Ba25 was not investigated in this study. However, it is acknowledged that PGE_2 is able to enhance the suppressive effect of Treg cells as well as induce a regulatory phenotype in $CD4^+$ $CD25^-$ T lymphocytes and this may have occurred.

IFN-γ
(ng/ml)

CHAPTER SEVEN:

EXPERIMENTAL SECTION : EFFECT OF Ba100 AND Ba25 ON RODENT MODELS OF TRANSPLANTATION – ALLOGRAFT SURVIVAL AND HISTOLOGICAL STUDIES

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1. Effect of Ba100 and Ba25 on allograft survival following heterotopic heart transplantation

1.1. Surgical procedure in brief

1.2. Results

2. Histological assessment following rat orthotopic renal transplantation in the absence or presence of Ba100 and Ba25

2.1. Surgical procedure in brief

2.2. Histological findings

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CHAPTER SEVEN:

EXPERIMENTAL SECTION : EFFECT OF BA100 AND BA25 ON RODENT MODELS OF TRANSPLANTATION – ALLOGRAFT SURVIVAL AND HISTOLOGICAL STUDIES

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The data from Chapters 5 and 6 shows that Ba100 and Ba25 inhibit T lymphocyte proliferation in response to mitogen, recall antigens and alloantigens. In summary, Ba100 inhibits the generation of lymphocytes expressing the activation markers HLA-DR and CD25, and downregulates the expression of the adhesion molecules CD11a, CD49d and CD29 and the costimulatory molecule CD28. Ba100 impairs cytokine production (IL-2, IL-4, IFN-γ and TNF-α) and prevents cell cycle progression and proliferating cell nuclear antigen expression. Ba25 has little effect on the generation of lymphocytes expressing activation markers, does not alter cell cycle progression nor PCNA expression, but generates an immunosuppressive Th2 cytokine profile. The immunosuppressive effects of Ba25 appear to be related to its ability to induce the generation of immunosuppressive prostaglandins. Thus, both proteins may well have *in vivo* immunomodulatory properties with the potential to prevent allograft rejection.

We therefore investigated whether Ba100 and Ba25 could prolong allograft survival in a rodent transplant rejection model and looked at their potential toxicity. All procedures were approved by the University of Cape Town Animal Ethics Committee and the rats were obtained from The University of Cape Town Animal Unit.

1. EFFECT OF BA100 AND BA25 ON ALLOGRAFT SURVIVAL FOLLOWING HETEROTOPIC HEART TRANSPLANTATION

We used a simplified model for heterotopic rat heart transplantation (Lee et al 1982).

BD IX and Long Evans rats weighing between 180 – 220 g were used. The rats were housed in an animal room at 22°C under a 12 h light – dark cycle and fed a commercial pelleted diet. The animals had free access to food and water.

1.1. Surgical procedure in brief

The donor heart was placed transversely in the right side of the abdominal cavity of the recipient. An end-to-side anastomosis of the donor aorta to the recipient's abdominal aorta was performed. The pulmonary artery was anastomosed end-to-side to the inferior vena cava. The heterotopic transplanted heart started beating immediately, with no period of fibrillation and a warm ischaemic period of less than 30 minutes. The circulation in the heterotopic transplanted heart was established through an *in vivo* coronary perfusion of the graft. The rats were assessed daily: i) General wellbeing ii) Abdominal palpitation to monitor the heart rate and the strength of contraction of the heterotopic heart transplant.

Ba100 (400 μl of a 200nM solution in PBS) and Ba25 (400 μl of a 200nM solution in PBS) were given intravenously via the tail vein of the rat, the day before the surgical procedure, during the operation just before unclamping the vessels and then on alternate days for 10 days.

All surgical procedures were performed under sterile conditions under ether anesthesia.

Heterotopic heart transplants were performed in the following groups and allograft survival time in days was measured.

Group 1: BD IX to BD IX recipients (non-rejecting model), n = 5.

Group 2: Long Evans to Long Evans recipients (non-rejecting model), n = 5.

Group 3: BD IX to Long Evans recipients (rejection model), n = 10.

Group 4: BD IX to Long Evans recipients + Ba100 (treatment model), n = 5.

Group 5: BD IX to Long Evans recipients + Ba25 (treatment model), n = 5.

* Control groups 1 and 2 were setup to establish a baseline allograft survival time in a non-rejecting heterotopic heart transplant model against which the allograft survival of the rejection model (group 3) and the treatment models (group 4 and 5) could be compared. The rats in these 2 control groups were killed between 90-120 days and were all healthy with functional heterotopic cardiac grafts. The rats in groups 3, 4 and 5 were killed when the heterotopic transplanted heart stopped beating.

1.2. Results

Table 1. Effect of Ba100 and Ba25 on allograft survival following heterotopic heart transplantation.

Rat model		Allograft survival in days	
		Range	Median
1.	BD IX to BD IX (non-rejecting model)	*Killed between 90 – 120 days	90
2.	Long Evans to Long Evans (non-rejecting model)	*Killed between 90 – 120 days	90
3.	BD IX to Long Evans (rejection model)	6 – 9 days	7
4.	BD IX to Long Evans + Ba100 (treatment model)	22– 68 days	26
5.	BD IX to Long Evans + Ba25 (treatment model)	18 – 24 days	24

The dosing regimen of Ba100 and Ba25 prolonged allograft survival following heterotopic cardiac allograft transplantation ($p = 0.001$, Mann-Whitney Non-parametric test). There was no evidence of any bleeding in the rats receiving either Ba100 or Ba25.

2. HISTOLOGICAL ASSESSMENT FOLLOWING RAT ORTHOTOPIC RENAL TRANSPLANTATION IN THE ABSENCE OR PRESENCE OF BA100 AND BA25

A rapid technique for renal transplantation in the rat was used (Engelbrecht et al 1992).

Sprague-Dawley (SD) and Dark Agouti (DA) rats weighing between 180 – 220 g were used. The rats were housed in an animal room at 22°C under a 12 h light – dark cycle and fed a commercial pelleted diet. The animals had free access to food and water.

2.1. Surgical procedure in brief

The left renal artery and vein of the recipient kidney were mobilised and the vessels divided as close as possible to the hilum of the kidney, the ureter was ligated and cut and the recipient kidney removed. The donor kidney was placed in the orthotopic position. The renal arterial anastomosis was performed using a modified sleeve technique. The renal vein was anastomosed end-to-end. The ureter was anastomosed to the bladder with the ureter tip being placed flush against the inside wall of the bladder. The warm ischaemic time was less than 15 minutes. The right kidney was removed from the recipient.

All surgical procedures were performed under sterile conditions under ether anaesthesia.

Ba100 (400 µl of a 200nM solution in PBS) and Ba25 (400 µl of a 200nM solution in PBS) were given intravenously via the tail vein of the rat, the day before the surgical procedure, during the operation just before unclamping the vessels and then on alternate days for 10 days.

Orthotopic renal transplants were performed in the following groups.

Group 1: SD to SD recipients (non-rejecting model), n = 5.

Group 2: DA to SD recipients (rejection model), n = 10.

Group 3: DA to SD recipients + Ba100 (treatment model), n = 5.

Group 4: DA to SD recipients + Ba25 (treatment model), n = 5.

Table 2. Rat survival following orthotopic renal transplantation.

	Rat model	Survival in days	
		Range	Median
1.	SD to SD (non-rejecting model)	All 5 survived > 100 days	
2.	DA to SD (rejection model)	3 – 8	7
3.	DA to SD + Ba100 (treatment model)	Killed on day 10 to assess histology	
4.	DA to SD + Ba25 (treatment model)	Killed on day 10 to assess histology	

Rats in Group 3 (Ba100) and Group 4 (Ba25) were killed on day 10 to histologically assess for rejection and signs of toxicity. The median survival in the rejection model was 7 days. The kidney, spleen, liver and lungs were removed from rats in groups 2, 3 and 4 for histological assessment.

2.2. Histological findings

Group 2: DA to SD (rejection model)

Kidney: Grade 3 T cell mediated rejection in 8 animals, grade 2 T cell mediated rejection in 2 animals.

Spleen: Reactive in all 10 animals.

Lungs: Congested in all 10 animals.

Liver: Normal in all 10 animals.

Group 3: DA to SD + Ba100

Kidney: Histology showed no rejection in 4 animals, grade 1 T cell mediated rejection in 1 animal

Spleen: Reactive in 1 animal.

Lungs: Normal in all 5 animals.

Liver: Normal in all 5 animals.

Group 4: DA to SD + Ba25

Kidney: Histology showed no rejection in 3 animals, grade 1 T cell mediated rejection in 1 animal, and a focal infarct, but no rejection in 1 animal.

Spleen: Reactive in 2 animals.

Lungs: Normal in all 5 animals.

Liver: Normal in all 5 animals.

This proof of concept rodent model of heterotopic cardiac allograft transplantation demonstrated that both Ba100 and Ba25 could prolong cardiac allograft survival.

Histological studies in the rat orthotopic kidney allograft model showed minimal or no rejection in the rats treated with Ba100 or Ba25.
Histological examination of the liver, lungs and spleen in the animals treated with Ba100 or Ba25 showed no evidence of haemorrhage, thrombosis or toxicity.

These proof of concept experiments demonstrated the *in vivo* immunosuppressive potential of Ba100 and Ba25. Ideal dosing regimens for optimal immunosuppression need to be established as well as their potential synergistic/additive immunosuppressive effects in combination with other known immunosuppressants.

CHAPTER EIGHT:

SUMMARY AND CONCLUSIONS

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We have isolated two novel C-type lectins, Ba100 and Ba25, from the venom of the Western Cape, South African puff adder, *Bitis arietans*. Both Ba100 and Ba25 modulate platelet function:

- Ba100 through its fibrinogenase activity prevents fibrinogen crosslinking and interferes with the formation of a completely cross-linked fibrin matrix, thereby inhibiting platelet aggregation and clot formation (Jennings et al 1999).

- Ba25 through its interaction with the GPIbα receptor of the von Willebrand receptor complex GPIb-IX-V and the collagen receptor $\alpha_2\beta_1$, leads to activation of the platelet fibrinogen receptor GPIIaIIIb and increased fibrinogen binding to the GPΠbIIIa receptor. Platelet agglutination in the absence of overall platelet activation occurs as a consequence of these interactions. Ba25 also inhibited collagen-induced expression of the platelet P-selectin (Jennings et al 2005; Jennings unpublished data).

Platelets play an integral role in inflammation by releasing pro-inflammatory factors and expressing cell surface receptors that bind to other blood cells, the extracellular matrix and the endothelium (Weyrich et al 2003; Kasirer-Friede et al 2007). Platelets are thus able to modulate intercellular interactions, adhesion and transendothelial migration of cells. Platelets and lymphocytes have a number of common integrin receptors including $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha v\beta_3$ and $\alpha_6\beta_1$, which mediate adhesive interactions between platelets, lymphocytes and the extracellular matrix. (Pribilia et al 2004; Kasirer-Friede et al 2007). Platelets have been shown to enhance T lymphocyte adhesion to the extracellular matrix (Solpov et al 2006; Shenkman et al 2006) and to play a role in the alloimmune response (Zhang et al 2003, 2005; Xu et al 2006).

The integrin receptor, $\alpha_2\beta_1$ is expressed on a variety of cells including platelets, mast cells, NK cells, activated T cells and endothelial cells (Shimizu et al 1999; Arase et al

2001; Sasaki et al 2003; Pribila et al 2004; Zutter & Edelson 2007; Kasirer-Friede et al 2007). The $\alpha_2\beta_1$ integrin acts as a receptor for a number of extracellular matrix ligands including collagen, laminin, E-cadherin and matrix-metalloproteinase-I and these interactions play an important role in inflammation (Zutter & Edelson et al 2007). $\alpha_2\beta_1$ also acts as a receptor for C1q complement protein and the collectins including the mannose-binding ligand and the surfactant protein A (Edelson at al 2006; Zutter & Edelson 2007). Therefore, the $\alpha_2\beta_1$ integrin plays a potentially important role in the innate immune response. (Edelson et al 2006; Zutter & Edelson 2007; McCall-Culbreath et al 2008).

The platelet collagen receptor $\alpha_2\beta_1$ plays an important role in the activation of the platelet fibrinogen receptor GPIIbIIIa and consequent platelet agglutination or aggregation depending on the level of signaling. GPIb-IX-V, GPVI and $\alpha_2\beta_1$ play synergistic roles in inducing platelet adhesion (Kuijpers et al 2003; Lecut et al 2004; Ozaki et al 2005).

The collagen binding $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins mediate lymphocyte adhesion to collagen (Hemler 1990) and are involved in inside-out and outside-in signaling to the lymphocyte. The $\alpha_2\beta_1$ integrin regulates the p38 MAPK pathway (Heino 2000). Lymphocyte $\alpha_2\beta_1$ integrin interaction with collagen type 1 has been shown to augment TCR-dependent expression and production of IFN-γ by T effector cells through activation of the ERK and JNK MAPK as well as the PI3-K/Akt activation pathways (Boisvert et al 2007). Lymphocyte $\alpha_2\beta_1$ also inhibits Fas-mediated apoptosis and caspase-8 activation through a protein phosphatase 2A-dependent activation of the MAPK/ERK pathway (Gendron et al 2003).

We thus postulated that our two novel C-type lectins, Ba100 and Ba25 that modulate platelet function either through their protease/fibrinogenase activity or through their targeting of platelet receptors, might be able to modulate T lymphocyte function.

The aim of our study was to assess the ability of Ba100 and Ba25 to inhibit T lymphocyte proliferative responses. We used standard lymphocyte proliferation assays used in assessing the function of known immunosuppressant agents (Hess & Tutschka 1980; Gelfand et al 1987; Bloemena et al 1989; Yoshimura et al 1989a,b; Dumont et al

1990b; Bierer et al 1991a) to investigate the potential immunosuppressive effects of the two novel C-type lectins, Ba100 and Ba25.

Both Ba100 and Ba25 inhibited in a dose-dependent manner calcium-dependent T lymphocyte proliferation in response to the mitogenic lectin PHA, several recall antigens and alloantigens participating in a mixed lymphocyte reaction. This was not due to cytotoxicity as the lymphocytes remained viable despite prolonged culture in the presence of Ba100 and Ba25.
Neither Ba100 nor Ba25 were able to inhibit calcium-independent T lymphocyte proliferation pathways such as proliferation in response to anti-CD28 antibody and a phorbol ester, PMA.

This inhibition of calcium-dependent, but not calcium-independent T lymphocyte proliferation pathways is similar to the inhibitory effects of the calcineurin inhibitors, Cyclosporin (Hess & Tutschka 1980; Fidelus & Laughter 1986; Gelfand et al 1987; Bloemena et al 1989; Ryffel 1990; Schreiber & Crabtree 1992) and FK506/Tacrolimus (Kino et al 1987b; Kay et al 1989a; Dumont et al 1990b; Bierer et al 1990a, 1991a; Johansson & Möller 1990), which are used in clinical practice to prevent allograft rejection.

Both Ba100 and Ba25, like Cyclosporin and FK506 (Hess & Tutschka 1980; Kay & Benzie 1984; Kay et al 1989b, 1990; Tocci et al 1989; Dumont et al 1990b; Metcalfe & Richards 1990; Henderson et al 1991) need to be added within the first 2 hours of the initiation of mitogen-induced PBMC proliferation in order to be effective and this suggests that Ba100 and Ba25 interfere with early events of T cell activation prior to DNA synthesis and cell division that involve calcium-dependent activation pathways leading to IL-2 production and IL-2 receptor expression. This is in contrast to Rapamycin, which acts late in T cell activation and inhibits both calcium-dependent and -independent T cell activation pathways (Sehgal & Bansbach 1993; Sehgal et al 1994; Wood & Bierer 1994; Sehgal 1995).

Proliferation in the presence of Ba100 at a concentration of 200 nM resulted in the inhibition of the generation of lymphocytes expressing the activation markers HLA-DR and CD25 and downregulated the expression of the adhesion/costimulatory molecules CD11a, CD49d and CD29 and the costimulatory molecule CD28 on lymphocytes.

These adhesion/costimulatory molecules together with the CD28 costimulatory molecule play a critical role in optimizing T lymphocyte activation through the bidirectional activation of antigen-presenting cells and T lymphocytes and the involvement of the CD28:B7 costimulatory pathway generating the second costimulatory signal (Salomon & Bluestone 2001; Sharpe & Freeman 2002; Nel 2002). Ba100 also inhibited the generation of T cell subsets that play a role as effector cells in the alloimmune response: the CTLs, NK cells and memory cells as well as increasing the CD4:CD8 ratio possibly reflecting the inability of CD8 lymphocytes to proliferate due to inhibition of the IL-2/IL-2 receptor pathway as Ba100 downregulated IL-2 receptor expression. The inhibitory effects of Ba100 and Ba25 on PHA-induced PBMC proliferation could not be reversed by the addition of exogenous IL-2, suggesting that both C-type lectins interfere with mitogen-induced T lymphocyte proliferation at more than one site. This is similar to the calcineurin inhibitors whose inhibitory activity is only partially reversed by exogenous IL-2 (Hess 1985; Gelfand et al 1987; Dumont et al 1990b; Lin et al 1991). Like Cyclosporin, Ba100 was able to inhibit the cytolytic activity of effector cells generated in a primary MLR (Hess & Tutschka 1980).

Both Ba100 and Ba25 were able to inhibit the proliferation of isolated CD4 and CD8 lymphocytes in the absence of antigen-presenting phagocytes suggesting that processing of these two C-type lectins by antigen-presenting phagocytes was not necessary for their immunosuppressive action.

Inhibition of proliferation of isolated CD4 lymphocytes in response to anti-CD3 antibody and the extracellular matrix protein, fibronectin was a characteristic of both Ba100 and Ba25. This may reflect the ability of these C-type lectins to either act as competitive antagonistic ligands for the fibrinogen receptors $\alpha_5\beta_1$ (VLA-5) and $\alpha_4\beta_1$ (VLA-4) or to downregulate receptor expression as was seen with Ba100 which was able to downregulate the expression of CD49d and CD29 adhesion molecules and thus impair cell-extracellular matrix interactions. Fibronectin plays an important role in the effector phase of the alloimmune response which is dependent on adhesion and migration of alloreactive cells into the graft (Kupiec-Weglinski et al 1993; Coito 1994a, 1998, 2000a) and therefore these inhibitory effects are potentially advantageous in limiting the alloimmune response.

Ba100 inhibited the production of Th1 cytokines (IL-2, IFN-γ, TNFα) and the Th2 cytokine IL-4 which play an important role in mediating allograft injury through delayed-type hypersensitivity reactions, promoting the generation of CTLs and the activation of NK cells and macrophages. This is similar to the effect of the calcineurin inhibitors (Hess et al 1982; Kronke et al 1984; Granelli-Piperno et al 1988a, 1990; Tocci et al 1989; Thomson et al 1995), but in contrast to rapamycin which has limited effects on cytokine production and instead blocks cytokine-mediated proliferative responses (Sehgal et al 1994; Wood & Bierer 1994; Bertagnolli et al 1994).

Like Cyclosporin and FK506/Tacrolimus (Metcalfe & Richards 1990; Chang et al 1991; Morris et al 1991), Ba100 inhibited lymphocyte cell cycle progression from the G0 – G1 phase into the S phase and this was associated with downregulation of the proliferating cell nuclear antigen, an obligate cofactor of DNA-polymerase-δ and a critical requirement for cell cycle progression into the S phase (Celis & Celis 1985; Maga & Hubscher 2003). Rapamycin prevents progression from the G_1 phase into the S phase of the cell cycle and blocks IL-2 induced PCNA gene expression (Terada et al 1993; Feuerstein et al 1995).

Although Ba25 inhibited T lymphocyte proliferative responses, a minimal change in the expression of T cell activation markers was observed. An immunosuppressive cytokine profile characterized by increased IL-4 and IL-10 production and decreased IFN-γ production was detected during a mixed lymphocyte reaction in the presence of Ba25 (200 nM). Ba25 did not affect cell cycle progression nor PCNA expression. The inhibitory effects of Ba25 on T lymphocyte proliferation appeared to be related to its ability to induce the generation of immunosuppressive prostaglandins. Indomethacin, a non-selective cyclooxygenase inhibitor added to PHA-induced PBMC proliferation reactions in the presence of Ba25, was able to reverse the Ba25-dependent inhibition of proliferation.

The immunosuppressive effects of Ba25 may be due to a combination of the immuno-inhibitory effects of prostaglandins: the direct inhibitory effects of prostaglandins on T lymphocyte proliferation (Choudhry et al 1999a,b; Ruggeri et al 2000; Vang et al 2001, 2003; Okano et al 2006), its ability to promote a Th2 cytokine profile (Snijdewint et al 1993; Katamura et al 1995; Hilkens et al 1996; Huang et al 1996, 1998; Stolina et al 2000; Pockaj et al 2004) and its effects on Tregs. The status of Tregs during

proliferation was not investigated, but PGE2 is able to enhance the suppressive effects of $CD4^+CD25^+$ Tregs as well as induce a regulatory phenotype in $CD4^+$ $CD25^-$ T lymphocytes (Baratelli et al 2005) and this may have occurred.

Cyclosporin, FK506 and Rapamycin can all modulate Tregs. Cyclosporin and FK506 inhibit induced Treg FOXP3 gene transcription (Baan et al 2005) and Cyclosporin inhibits the expansion of $CD4^+$ $CD25^+$ Tregs (Lim et al 2007) following alloantigen stimulation. Clinically, Cyclosporin has been shown to inhibit the generation of $CD4^+$ $CD25^+$ CTLA-4^+ and $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs in renal transplant recipients (Korczak-Kowalska et al 2007).
Rapamycin has been shown to selectively promote the expansion of functional human $CD4^+$ $CD25^+$ $FOXP3^+$ Tregs and deplete $CD4^+$ $CD25^-$ T effector cells (Battaglia et al 2005, 2006; Strauss et al 2007).

We did not investigate the biochemical targets and molecular mechanisms of action involved in the inhibition of T lymphocyte proliferative responses by Ba100 and Ba25. Possible molecular mechanisms of action in view of their C-type lectin structure include:

i) acting as competitive ligands for the lymphocyte integrin receptors LFA-1 and the β1 integrins $\alpha_4\beta_1$ and $\alpha_2\beta_1$, thereby preventing inside-out and outside-in signaling and the formation of the mature immunological synapse which is needed for effective TCR-mediated T cell activation (Hogg et al 2003) as well as preventing cell-extracellular matrix interactions involved in cellular activation, adhesion and migration. This may be mediated by the binding of Ba100 and Ba25 to the Lewis x trisaccharide known to be the binding site for other C-type lectins including the selectins (Sanders et al 1996), dendritic cell lectin receptors such as DC-SIGN (Engering et al 2002; Van Liempt et al 2004) and the scavenger receptor C-type lectin (Nakamura et al 2001; Coombs et al 2005).

ii) preventing optimal antigen-presenting cell including dendritic cell function by binding to their lectin receptors involved in antigen presentation and the initiation of T lymphocyte activation.

The demonstrated immunosuppressive effects of Ba100: Inhibition of calcium-dependent T lymphocyte proliferation associated with the downregulation of activation markers (HLA-DR and CD25) and adhesive/costimulatory molecules (CD11a, CD49d, CD29 and CD28), together with the inhibited generation of critical T cell subsets (CTLs, NK cells and memory cells) involved in the alloimmune response and the impaired production of Th1 cytokines and IL-4 that drives the alloimmune response leading to allograft injury suggest a potential therapeutic role for Ba100 as an immunosuppressive agent.

Ba25 similarly affected calcium-dependent T lymphocyte proliferation, but did not modulate the activation markers nor cell cycle progression. Instead, Ba25 generated an immunosuppressive Th2 cytokine profile possibly through the generation of immunosuppressive prostaglandins. Thus, the immunosuppressive effects of Ba25 may be due to a combination of direct inhibitory effects of PGE2 on T lymphocyte proliferation, the ability of PGE2 to promote a Th2 cytokine profile and to enhance the suppressive effects of Tregs as well as induce a regulatory phenotype in $CD4^+$ $CD25^-$ T lymphocytes. The potential ability of Ba25 to enhance Treg function without interfering with T lymphocyte activation suggests a potential role for Ba25 as an agent capable of promoting immune tolerance.

Thus, the ability of Ba100 and Ba25 to modulate platelet function and inhibit calcium-dependent T lymphocyte proliferation, both critical components of the alloimmune response leading to allograft injury, suggests a potential therapeutic role for these two novel C-type lectins. Proof of concept rodent models of allograft rejection demonstrated that both Ba100 and Ba25 could prolong allograft survival without any evidence of haemorrhage, thrombosis or toxicity. Thus, Ba100 and Ba25 may well prove after indepth analysis of their structure-function relationships to be capable of being developed as therapeutic agents for the prevention of allograft rejection.

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APPENDIX ONE:

MATERIALS AND METHODS

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APPENDIX ONE:

MATERIALS AND METHODS

1. Materials

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1.1. Reagents

All chemicals and reagents were obtained from Sigma Chemical Co (St Louis, MO), except for those listed below:

RPMI 1640 medium (Gibco, The Scientific Group/Adcock-Ingram, RSA), AB serum and fresh frozen plasma (Western Province Blood Transfusion Service, RSA), Serum-free culture medium (CG medium from Serotec), Bovine serum albumin (Seravac Biotech Pty (Ltd), Epping, RSA), Penicillin (Gibco, The Scientific Group/Adcock-Ingram, RSA), Streptomycin (Gibco, The Scientific Group/Adcock-Ingram, RSA), Phytohaemagglutinin/PHA (Murex Diagnostics Ltd., Dartford, England), PPD - a purified protein derivative of Mycobacterium tuberculosis (Central Veterinary Laboratory, Weybridge, UK), Tetanus toxoid (Lederle Laboratory, Wayne, NJ), Varidase (Lederle Laboratory, Wayne, NJ), Recombinant human Interleukin-2 (10^7 U/mg, Pharmingen, San Diego, CA), Dynabeads M-450 CD4 and CD8 and Detachabeads for isolation of $CD4^+$ and $CD8^+$ lymphocytes (Dynal, Southern Cross Biotechnology, Claremont, RSA), Tritiated thymidine and ^{51}Chromium (Amersham Corporation,UK), DNA-Prep (Coulter Corporation, Maine, Florida) - a Coulter reagent kit with cell lysing solution, permeabilising solution and DNA staining reagent was used to assess progression through the cell cycle using flow cytometry.

1.2. Antibodies

1.2.1. Antibodies for phenotypic analysis of lymphocytes

The following FITC-labelled monoclonal antibodies used for phenotypic analysis of lymphocytes following PHA and alloantigen (MLR) stimulation were purchased from Immunotech, a Coulter Company (Marseille, France).

i) Monoclonal antibodies (IgG1 and IgG2a mouse) used as isotypic controls.

ii) Monclonal antibodies (IgG1κ mouse) to CD3, CD4, CD56, HLA-DR, CD11a, S6F1, CD19.

iii) Monoclonal antibodies (IgG2a mouse) to CD29.

iv) Monoclonal antibodies (IgG1 mouse) to CD8, CD28 and CD49d.

v) Monoclonal antibody (IgG2a rat) to CD2.

vi) Monoclonal antibody (IgG2aκ mouse) to CD25.

1.2.2. Antibodies for measurement of lymphocyte intracellular cytokine production in response to PMA and ionomycin in the absence or presence of Ba100

The following FITC- or R-Phycoerythrin-labelled monoclonal antibodies for measuring intracellular cytokine production by flow cytometry were purchased from Pharmingen (San Diego, CA): FITC-rat anti-human IL-4, FITC- mouse anti-human TNF-α, FITC-mouse anti-human IFN- , FITC-mouse anti-human IgG1 isotype control immunoglobulin, R-PE-rat anti-human IL-2, R-PE-rat IgG2a isotype control immunoglobulin.

1.2.3. Antibodies for measurement of cytokine production by activated lymphocytes in the MLR in the absence or presence of Ba25

ELISA assay kits were purchased from Pharmingen (San Diego, CA) for measurement of IL-4, TNF-α and IFN- production.

1.2.4. Antibodies used to assess PCNA expression by flow cytometry

The following Coulter clone monoclonal antibodies were purchased from Coulter Electronics: PCNA (IgG1 and IgM), Coulter clone isotypic controls (MsIgG1, MsIgM)

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1.2.5. Antibodies used in OKT3/fibronectin stimulated CD4 proliferation assays and in calcium-independent PBMC proliferation assays

i) OKT3 (orthoclone OKT3, a muromonoclonal anti-CD3 antibody from Ortho Pharmaceuticals Corporation, Raritan, NJ), ii) anti-CD28 antibody (monoclonal IgG2b rat from Serotec).

2. Collection of blood and lymphocyte isolation

Peripheral blood mononuclear cells (PBMC), $CD4^+$ and $CD8^+$ T lymphocytes were isolated from venous blood donated by healthy adult volunteers. Blood was collected into 10ml lithium heparinised vacutainer tubes.

2.1. Isolation of human peripheral blood mononuclear cells (PBMC)

PBMC were isolated from heparinised venous blood by Ficoll-Hypaque density-layer centrifugation. The isolated cells contained > 95% mononuclear cells and viability of the cells was > 95% as judged by trypan blue exclusion.

PBMC were washed x3 and resuspended in complete culture medium containing RPMI-1640 supplemented with 10% heat inactivated human AB serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) at a concentration of $1x10^6$ cells/ml for use in PBMC proliferation assays unless otherwise stated.

2.2. Isolation of $CD4^+$ and $CD8^+$ lymphocytes from whole blood

Immunomagnetic cell isolation using Dynabeads M-450 coated with a monoclonal antibody specific for CD4 or CD8 membrane antigens was used to isolate $CD4^+$ and $CD8^+$ T cells (Leivestad et al 1987) according to manufacturer's instructions. Briefly the $CD4^+$ and $CD8^+$ cells were isolated from the mononuclear cell layer obtained from heparinised whole blood after Ficoll-hypaque density-layer centrifugation. Following a short incubation period, the $CD4^+$ and $CD8^+$ T cells bind to the Dynabeads. The positively selected cells are then detached from the Dynabeads using appropriate detachabeads. The isolated cells are > 99% pure, > 98% viable and are not activated by the process.

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3. Isolation of Ba100 and Ba25

The 2 novel C-type lectins were isolated from the venom of the Western Cape, South African puff adder, *Bitis arietans* using fibrinogen and platelet receptor affinity chromatography respectively and provided for the project by Mr. Brent Jennings (Jennings et al 1999, 2005).

3.1. Gel filtration

Lyophilised crude venom (100 mg), from puff adders found in the Western Cape region of South Africa, was dissolved in 3 ml 0.1 M acetic acid (glacial, Riedel-deHaën), clarified by centrifugation at 3000 rpm, 10 min, then applied to a Toyopearl (Tosohaas) HW-50 column (1.8 cm x 70 cm) equilibrated in 0.1 M acetic acid. Fractions (2 ml) were eluted from the column in the same buffer and protein was detected by absorbance at 280 nm (Hitachi U-2000 spectrophotometer) (Fig. 1A). The nature of proteins in each fraction was determined by mixing an aliquot with one-third volume of three-fold strength SDS-sample buffer containing 0.01 M Tris (pH 6.8), 6% SDS and 36% glycerol and analysed on 10% SDS-PAGE. Fractions were combined as indicated in Fig. 1A to give pools A, B and C, then lyophilised and redissolved in 10 ml buffer A: 10mM Tris, pH 7.4 with 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, followed by dialysis in buffer A. The nature of the proteins in pools A, B and C was determined by analysis on 10% SDS-PAGE (Fig. 1B).

3.2. Affinity chromatography

3.2.1. Isolation of Ba100

A fibrinogen-affinity column was made by coupling purified human fibrinogen (isolated by $(NH_4)_2$ SO_4 precipitation of plasma (Kalvaria et al 1986)) to cyanogen bromide-activated Sepharose CL4B (Sigma) according to manufacturer's instructions and equilibrated with buffer A. The presence of fibrinogen binding proteins in Pools A, B and C (Fig. 1A) were investigated by passing the individual pools over the fibrinogen affinity column. Protein bound to the column was eluted in 1 ml fractions with 0.1 M sodium acetate buffer, pH 4.0, containing 1 M NaCl. Fractions containing protein as

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assessed by measuring the absorbance at 280 nm were pooled, then dialysed immediately afterwards into one-tenth strength phosphate buffered saline (PBS) and then lyophilised and redissolved in Milli-Q filtered water (Millipore) to one-tenth original volume. Protein concentration was determined by the method of Lowry and the nature of the isolated protein assessed by analysis on 10% SDS-PAGE in the presence and absence of a reducing agent. A fibrinogen binding protein with an apparent molecular mass of 100 kDa in the absence of reducing agent was isolated from both Pool A and Pool B and was called Ba100 (Fig. 2). No fibrinogen binding protein was found in pool C.

Subsequently, Ba100 was isolated by fibrinogen affinity chromatography from a pool of A and B (Fig. 1A). The eluted protein was dialysed into one-tenth strength phosphate buffered saline (PBS), then lyophilised and redissolved in Milli-Q filtered water (Millipore) to one-tenth original volume. The purity and apparent molecular mass of the protein in each batch of Ba100 that was prepared, was confirmed using 10% SDS-PAGE in the absence of reducing agent. Protein concentration of the batches of Ba100 was determined by the method of Lowry and then adjusted with sterile PBS to a final concentration of 1mg/ml before use in cell culture experiments.

3.2.2. Isolation of Ba25

The presence of GPIIbIIIa binding proteins in Pools A, B and C (Fig. 1A) were investigated using a GPIIbIIIa affinity column. This column was prepared by coupling a preparation of fibrinogen depleted (using a fibrinogen affinity column prepared as described above) GPIIbIIIa receptor, isolated from outdated platelets (Fitzgerald et al 1985) to Sepharose CL-4B. The protein in pools A, B and C, (Fig. 1A) were dialysed into Tris saline buffer (TS150 C/M, 10mM Tris pH 7.4, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$) before being loaded onto a GPIIbIIIa-affinity column, after which the column was washed with 10 column volumes of TS150 C/M. Bound protein was eluted with 0.1 M sodium acetate buffer, pH 4, containing 1 M NaCl. Protein in each fraction (1 ml) was estimated by measuring the absorbance at 280 nm. Peak fractions were pooled and immediately dialysed into one-tenth strength PBS, lyophilised and redissolved in Milli-Q filtered water (Millipore) to one-tenth original volume. Protein concentration was determined by the method of Lowry and the nature of the isolated

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protein assessed by analysis on a 5-13% SDS-PAGE in the presence and absence of a reducing agent. Only Pool C (Fig 1A) was found to contain a protein with an apparent molecular mass of 25 kDA that bound to the GPIIbIIIa affinity column and was called Ba25 (Fig. 3).

Subsequently, Ba25 was isolated by GPIIbIIIa affinity chromatography from Pool C (Fig. 1A). The eluted protein was dialysed into one-tenth strength phosphate buffered saline (PBS), then lyophilised and redissolved in Milli-Q filtered water (Millipore) to one-tenth original volume. The purity and apparent molecular mass of the protein in each batch of Ba25 that was prepared was confirmed using 5-13% SDS-PAGE in the absence of reducing agent. Protein concentration of the batches of Ba25 was determined by the method of Lowry and then adjusted with sterile PBS to a final concentration of 0.25 mg/ml before use in cell culture experiments.

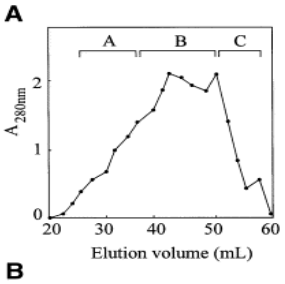

Fig. 1 (A): Gel filtration of crude venom on HW-50 column.

100 mg crude venom was dissolved in 3 ml 0.1 M acetic acid, then applied to a Toyopearl HW-50 column (1.8 cm × 70 cm) at 0.5 ml/min and eluted with the same buffer. 2 ml fractions were collected at 4°C. Protein was detected by absorbance at 280 nm.

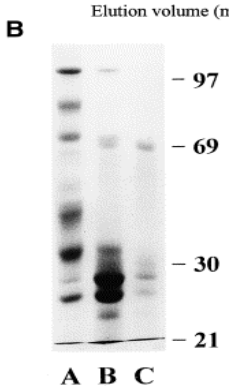

Fig. 1(B): Nature of the proteins in pools A, B and C.

Fractions eluting off the HW-50 column were pooled as described for isolating Ba100 and Ba25 and analysed on 10% SDS-polyacrylamide gels under non-reducing conditions. Proteins were stained by Coomassie blue. Molecular masses in kDa are indicated on the right of the figure.

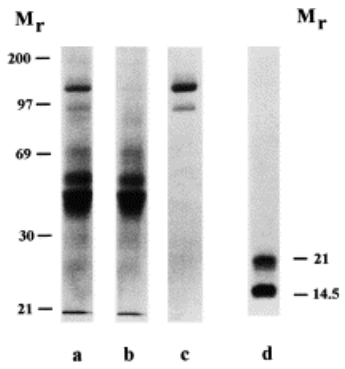

Fig. 2. Fibrinogen-affinity chromatography isolation of Ba100. Pools A and B (Fig. 1 A), obtained from gel filtration of crude venom, were combined, lyophilised and re-dissolved in buffer A and then passed over a fibrinogen-affinity column as described in the methods. 10% SDS-PAGE of: (a) proteins in pools A and B prior to affinity column treatment, (b) nature of proteins not bound to the fibrinogen column, (c) nature of Ba100 eluted from the affinity column, (d) nature of Ba100 under reducing conditions. Relative molecular masses (in kDa) are shown.

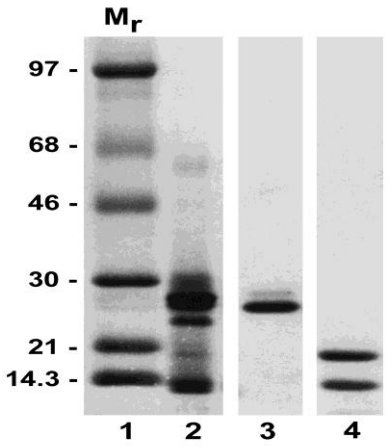

Fig. 3. GPIIbIIIa affinity chromatography isolation of Ba25. Pool C (Fig. 1A) obtained from gel filtration of crude venom was lyophilized, re-dissolved in buffer A and then passed over the GPIIbIIIa affinity column as described in the methods. SDS-PAGE analysis (5–13% polyacrylamide gradient gel) of: Lane 1, molecular weight markers (weights in kDa on the left); Lane 2, protein from pool C prior to loading on the GPIIbIIIa column; Lane 3, Protein eluted from the affinity column called Ba25; Lane 4, Ba25 in the presence of a reducing agent.

4. Preparation of fibronectin

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Fibronectin (Fn) isolated from 500ml aliquots of fresh frozen plasma obtained from the Western Province Blood Transfusion Service was a protein regularly prepared in the laboratory and provided for the project. 5mM benzamidine was added to the plasma immediately after centrifugation and plasma was filtered (Whatman's No.1 filter paper) to remove insoluble lipid.

4.1. Gelatin-sepharose affinity chromatography

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The non-denaturing affinity chromatography method of Vuento and Vaheri (1979) was routinely used to prepare fibronectin.

Gelatin-sepharose affinity matrix was prepared by coupling gelatin to cyanogen bromide (CNBr)-activated Sepharose 4B according to the manufacturer's instructions.

5mM benzamidine was added to all buffers to prevent proteolysis and 0.02% sodium azide was added to the buffers to prevent bacterial contamination.

A 1.5 x 13 cm column (Amicon) loaded with gelatin-sepharose affinity matrix was equilibrated with equilibrating buffer (0.05M Tris-HCI, pH 7.5). 50 ml plasma was loaded and washed through the column with equilibrating buffer until the UV monitor recording (280 nm) returned to zero. 1M NaCl was added to the equilibration buffer and the column washed again. Fibronectin was then eluted with 2M arginine in equilibrating buffer without 1M NaCl in 1ml aliquots. The column was regenerated with 8M urea.

The eluate fractions containing the protein peak were analysed by SDS-PAGE (Laemmli, 1970). The protein peak was dialysed into phosphate buffered saline (PBS), pH 7.3 using a Spectrapor 4 dialysis membrane (MWT cut-off 12-14 000) and the protein concentration was determined by means of the Biorad method (Bradford, 1976)

5. Preparation of OKT3-coated wells/microtitre plates

200 µl of OKT3, a muromonoclonal anti-CD3 antibody at a concentration of 100 ng/ml in 0.01M PBS, pH 7.4 was pipetted into each well of a 96-well flat-bottomed microtitre plate, incubated at room temperature for 3 h and then kept overnight at 4°C. The following day, the microtitre plate was washed x2 with PBS and used in the CD4 lymphocyte proliferation assays (Matsuyama et al 1989).

6. Measurement of tritiated thymidine ^{3}[H] incorporation to assess lymphocyte proliferation

After the prescribed length of culture, 1 µCi of [^{3}H]-thymidine was added to each microtitre plate well and cultures incubated for a further 18 h before harvesting onto glass fibre filter paper with a multiple automated sample harvester. The filter papers were dried and processed for liquid scintillation counting.

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7. Lymphocyte proliferation assays

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7.1. PBMC proliferation assays

7.1.1. PBMC proliferation assays in response to mitogen and antigens

PBMC ($1x10^5$ cells/well) were cultured in triplicate with the mitogen, Phytohaemagglutinin (PHA 0.00575 mitogenic units) in a 96-well round-bottomed microtitre plate in the absence or presence of Ba100 or Ba25 in a humidified 5% CO_2 in air incubator at 37°C. Ba100 or Ba25 was pre-incubated with PBMC for 30 min at 37°C prior to the addition of PHA. Ba100 or Ba25 was used at the following final concentrations: 200, 100, 50, 25 or 12.5 nM as stated in the results section. At 72 h, 1 μCi [^{3}H] thymidine was added to each well and incorporation measured 18 h later.

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The effect of preincubation with Ba100 or Ba25 for varying periods (0, 30 and 60min preincubation) on PHA-induced PBMC proliferation was assessed. Pre-incubation with Ba100 or Ba25 did not influence the inhibitory effect on lymphocyte proliferation and for standardization purposes, a 30 minute pre-incubation period was used for all subsequent experiments unless otherwise stated. The effect of preincubating Ba100 or Ba25 with PBMC for 30 min at 37°C, then washing it away before the addition of PHA to initiate proliferation, was also investigated.

The experiments assessing the effect of various concentrations of Ba100 or Ba25 on PBMC proliferation were repeated using the following recall antigens:

i) PPD, a purified protein derivative of Mycobacterium tuberculosis at a final concentration of 3 μg/ml.

ii) Tetanus toxoid antigen (formol toxoid, stock concentration 10 Lf/0.5 ml) at a final concentration of 1:20.

iii) Varidase (streptokinase – streptodornase: 250 U/ml streptokinase, 62.5 U/ml streptodornase) at a final concentration of 1:40.

7.1.2. PBMC proliferation assays in flasks

These assays were setup to assess the effect of Ba100 or Ba25 on

i) Lymphocyte intracellular cytokine production in response to PMA and ionomycin stimulation.

ii) Cell cycle progression and PCNA expression in PHA-stimulated PBMC.

Isolated PBMC were suspended at $1x10^6$ cells/ml RPMI-1640/10% AB serum in 30 ml tissue culture flasks. PBMC ($20x10^6$/20 ml RPMI-1640/10% AB serum) were cultured with the stimulant in the absence or presence of Ba100 (200 nM) or Ba25 (200 nM) in a humidified 5% CO_2 in air incubator at 37°C. Ba100 (200 nM) or Ba25 (200 nM) was preincubated with PBMC for 30 min at 37°C prior to the addition of the stimulant. At 72 h, proliferation was measured by counting the number of cells/ml or by standard [^{3}H] thymidine incorporation to confirm the inhibitory effect of Ba100 or Ba25.

7.1.3. Primary mixed lymphocyte reaction (micro-MLR)

PBMC were isolated by Ficoll-Hypaque density centrifugation. A primary mixed lymphocyte reaction was initiated by adding $1x10^5$ irradiated (3000R) stimulator cells to $1x10^5$ responder cells (each in a volume of 0.05 ml) per well in a 96-well round-bottomed microtitre plate and incubated in a humidified 5% CO_2 in air incubator at 37°C. Responder cells were preincubated with Ba100 or Ba25 in varying concentrations (12.5 – 200 nM) for 30 min at 37°C prior to the addition of the stimulator cells. In the control primary MLR, the responder cells were not exposed to Ba100 or Ba25. On day 6, 1 μCi [^{3}H] thymidine was added to each well and incorporation was measured 18 h later.

7.1.4. Bulk MLR

Bulk MLRs were setup in 30ml tissue culture flasks for

i) The determination of T cell subsets and phenotypic analysis of effector cells generated in a primary MLR in the absence or presence of Ba100 or Ba25.

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ii) Assessment of suppressor cell activity of primary MLR generated effectors on a fresh MLR and the cytolytic activity of primary MLR generated effectors.

Responder cells ($1x10^6$ cells/ml) were cultured with $1x10^6$ cells/ml irradiated (3000R) stimulator cells in the absence or presence of Ba100 (200 nM) or Ba25 (200 nM) in a humidified 5% CO_2 in air incubator at 37°C . Ba100 (200 nM) or Ba25 (200 nM) was pre-incubated with the responder cells for 30 min at 37°C prior to the addition of the stimulator cells. On day 6, proliferation was assessed either by counting the number of cells/ml or by pipetting 100 µl cell suspension into triplicate wells of a microtitre plate, adding 1 µCi [^{3}H] thymidine to each well and measuring incorporation 18 h later.

7.1.5. PBMC proliferation in response to anti-CD28 antibody and a phorbol ester, phorbol 12-myristate 13-acetate (PMA)

PBMC ($0.5x10^6$ cells/well) in 1% BSA/PBS were cultured in triplicate with PMA (1 ng/ml); PMA (1 ng/ml) + anti-CD28 antibody (1 ng/ml) and PMA + anti-CD28 antibody + Ba100 (200 nM) or Ba25 (200 nM) in a humidified 5% CO_2 in air incubator at 37°C. Ba100 or Ba25 was pre-incubated with PBMC for 30 minutes at 37°C prior to the addition of PMA or PMA + anti-CD28 antibody. At 72 h, 1 µCi [^{3}H] thymidine was added and incorporation measured 18 h later.

7.1.6. Addition of exogenous IL-2 to PHA-induced PBMC proliferation assays

PBMC ($1x10^5$ cell/well) were cultured in triplicate with PHA (0.00575 mitogenic units); PHA + Ba100 (200 nM) or Ba25 (200 nM); PHA + IL-2 (10 U/ml) and PHA + Ba100 or Ba25 + IL-2 in a humidified 5% CO_2 in air incubator at 37°C. Ba100 or Ba25 was preincubated with PBMC for 30 minutes at 37°C prior to the addition of PHA or PHA + IL-2. At 72 h, [^{3}H] thymidine was added and incorporation measured 18 h later.

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7.2. CD4 and CD8 lymphocyte proliferation assays

7.2.1. CD4 and CD8 lymphocyte proliferation in response to PHA

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$CD4^+$ and $CD8^+$ lymphocytes were isolated from PBMC using Dynabeads as described above and then reconstituted in a 60 : 40% CD4 : CD8 ratio (ratio found in whole blood).

$1x10^5$ $CD4^+$ and $CD8^+$ lymphocytes/well were cultured in triplicate with PHA (0.00575 mitogenic units) or PHA + Ba100 or Ba25 in varying concentrations (as stated in the results section) in a humidified 5% CO_2 in air incubator at 37°C. The $CD4^+$ and $CD8^+$ lymphocyte suspension was pre-incubated with Ba100 or Ba25 for 30 min at 37°C prior to the addition of PHA. At 72 h, 1 μCi [^{3}H] thymidine was added and incorporation measured 18 h later.

7.2.2. CD4 lymphocyte proliferation in response to OKT3 and fibronectin

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$CD4^+$ lymphocytes were isolated from PBMC using Dynabeads. $1x10^5$ $CD4^+$ lymphocytes/well were cultured in triplicate in an OKT3-coated 96-well flat-bottomed microtitre plate in serum-free culture medium (Serotec medium) with or without fibronectin (30 μg/ml) in the absence or presence of Ba100 (200 nM) or Ba25 (200 nM) in a 5% CO_2 in air incubator at 37°C. The CD4 lymphocytes were pre-incubated with Ba100 or Ba25 for 30 min at 37°C prior to the addition of fibronectin to the wells. 1 μCi [^{3}H] thymidine was added on days 1, 2 and 4 and incorporation measured 18 h later.

7.3. Suppressor cell assay

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Assessment of suppressor cell activity of effector cells generated in a primary bulk MLR was performed by using a slight modification of the technique described by Hirschberg and Thorsby (1977).

A bulk primary MLR was set up by incubating responder cells, A ($1x10^6$ cells/ml) with irradiated (3000 R) stimulator cells, Bx ($1x10^6$ cells/ml) in complete media in the absence or presence of Ba100 (200 nM) or Ba25 (200 nM) in a humidified 5% CO_2 in air incubator at 37°C. On day 6, the cells were harvested, incubated for 30 min, washed

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x 2 in fresh complete media to remove Ba100 or Ba25, and used as the primed cells in the suppressor cell assay.

Fresh MLR reactions contained the component cells A+Bx, C+Ex, C+Bx, A+Cx: $1x10^5$ responder cells (A or C) were cultured in triplicate with $1x10^5$ irradiated (3000 R) stimulator cells (Bx, Cx or Ex) per well in complete media in a 96-well round-bottomed plate at 37°C.

These fresh MLR (A+Bx, C+Ex, C+Bx and A+Cx) were cultured with the primed cells in the following combinations.

1. Primed cells autologous to responders and stimulators. 2. Primed cells, responders and stimulators completely heterologous. 3. Same stimulator, different responder. 4. Same responder, different stimulator.

On day 6, [^{3}H] thymidine was added and incorporation measured 18 h later.

7.4. Measurement of cell mediated lympholytic (cytotoxic) activity of the MLR generated effectors in a ^{51}Cr release assay

A bulk primary MLR (A+Bx) was set up in the absence and presence of Ba100 (200 nM). After 6 days culture, primed cells were harvested from bulk MLR cultures and used as effectors in the cell-mediated lympholysis assay. Primed cells harvested from the bulk MLR were incubated for 30 min, washed x2 in fresh complete media to remove Ba100 before assessment of cell mediated lympholytic activity.

$1x10^4$ 3-day-old PHA blast cells (prepared from donor B) labeled with ^{51}Cr (100 µCi ^{51}Cr/$2x10^6$ target cells) served as targets.

The effectors and targets were incubated in 96-well round-bottomed microtitre plates in the following effector: target ratios: 40 : 1, 20 : 1 and 10 : 1 in a final volume of 200 µl. Before incubation for 4 h at 37°C, the plates were subjected to light centrifugation (100 G, 1 min). At the end of the incubation period, the plates were centrifuged (500 G, 5 min) and 100 µl supernatant was removed and counted for released ^{51}Cr. Maximum and spontaneous ^{51}Cr release was obtained by adding 0.1 ml 1N HCl or 0.1 ml medium respectively to wells containing $1x10^4$ target cells.

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% specific ^{51}Cr release was calculated according to the following formula:

$$\% \text{ specific } {}^{51}\text{Cr release} = \frac{\text{DPM experimental} - \text{DPM spontaneous}}{\text{DPM maximum} - \text{DPM spontaneous}} \times 100$$

8. Lymphocyte populations and phenotype after proliferation of PBMC in response to PHA in the absence or presence of Ba100 or Ba25

Flow cytometry was used to analyse the phenotype of the lymphocyte population after proliferation of PBMC in response to PHA in the absence or presence of Ba100 (200 nM) or Ba25 (200 nM). Both the % of cells in the lymphocyte gate (set on lymphocyte forward and side scatter properties) expressing a particular receptor and the MFI (mean fluorescent intensity) of the antibody binding was determined.

8.1. Methods of antibody labeling

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Cells were harvested at the end of PHA-induced PBMC proliferation assays. 10 µl of each antibody to be tested was added per well of a V-bottomed plate. Harvested cells were resuspended and pipetted with mixing into the V-bottomed wells containing the antibody. The V-bottomed plate containing the cell suspension and labeling antibodies was incubated for 15 min in a dark cupboard and then spun at 2000 rpm for 4 min at 2 - 8°C. The supernatant was removed and 200 µl PBS/10% AB serum was added to each well and mixed well. The plate was then spun at 2000 rpm for 4 min at 2 – 8°C, the supernatant removed and 100 µl PBS/10% AB serum added.100 µl 2% paraformaldehyde was added to Kimble tubes, the cell suspension was added and mixed. A further 100 µl 1% paraformaldehyde was then added to the Kimble tubes. Single colour analysis of antibody binding was done by flow cytometry (EPICS XL flow cytometer, Beckman Coulter) on gated lymphocytes. Both the percentage of cells in the lymphocyte gate expressing the receptor and the MFI was measured.

The receptors analysed included:

i) Activation markers: HLA-DR and CD25 (α chain of IL-2 receptor)

ii) Adhesion receptors: CD2, CD11a, CD49d and CD29

iii) Costimulatory molecules: CD28

iv) CTL markers: S6F1

v) NK cell markers: CD56 and CD16

vi) B cell markers: CD19

vii) Anti-CD3 to detect T cells

viii) Anti-CD4 and anti-CD8 to determine T cell subsets

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9. Lymphocyte populations and phenotype of effector cells generated in a 6 day primary MLR in the absence or presence of Ba100 or Ba25

A bulk primary MLR in the absence or presence of Ba100 (200 nM) or Ba25 (200 nM) was set up in 30 ml tissue culture flasks. $1x10^6$/ml responder cells were cultured with $1x10^6$/ml irradiated (3000R) stimulator cells in the absence or presence of Ba100 (200 nM) or Ba25 (200 nM) in a humidified 5% CO_2 in air incubator at 37°C. Ba100 (200 nM) or Ba25 (200 nM) was pre-incubated with the responder cells for 30 min at 37°C prior to the addition of the stimulator cells. On day 6, cells were harvested, cell proliferation assessed by counting the number of cells/ml. Cells were labelled as described for the PHA-stimulated PBMC proliferation assays with antibodies directed to the indicated lymphocyte receptors and single colour analysis of antibody binding was done by flow cytometry on gated lymphocytes. Both the percentage of cells in the lymphocyte gate expressing the receptor and the MFI was measured.

10. Measurement of cytokine production

Cytokine production by activated lymphocytes was determined by using:

i) Flow cytometry to detect intracellular cytokine production by PBMC in response to PMA and ionomycin in the absence or presence of Ba100.

ii) Immunoassays to assess cytokine production during a primary mixed lymphocyte reaction in the absence or presence of Ba25.

10.1. Intracellular cytokine production by PBMCs after PMA and Ionomycin stimulation (method according to Sander et al 1991).

PBMC ($20x10^6$/20 ml RPMI 1640/10% AB serum were cultured in 30 ml tissue culture flasks with PMA (50 ng/ml), ionomycin (1 μM) and 2.5 μg/ml Brefeldin (an inhibitor of intracellular protein transport process) in the absence or presence of Ba100 (200 nM) in a humidified 5% CO_2 in air incubator at 37°C .

At varying time points (4, 6 and 24 h of stimulation), stimulated PBMC were harvested and the concentration adjusted to $10x10^6$ cells/ml RPMI 1640/10% AB serum. 100 μl cell suspension aliquots were fixed with 4% Paraformaldehyde and permeabilised with 0.1% Saponin. The cells were then labelled with antibodies to IL-2, IL-4, TNF-α and IFN-γ. Single colour analysis of cytokine antibody binding was done by flow cytometry on 10 000 gated $CD3^+$ lymphocytes. The percentage of cells in the lymphocyte gate expressing the cytokine as well as the mean fluorescent intensity of the cytokine antibody binding was measured.

The kinetic study was performed, harvesting cells at 4, 6 and 24 h to establish the optimal time point for measuring the intracellular IL-2, IL-4, IFN-γ and TNF. The optimal time point was 4 h.

Standard ELISA kits (Pharmingen, San Diego, CA) were utilised according to instructions to measure IL-4, IL-10 and IFN-γ production during a mixed lymphocyte reaction in the absence or presence of Ba25 (200 nM)

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11. Assessment of the distribution of PHA-stimulated PBMC in the various phases of the cell cycle

PHA-stimulated PBMC proliferation assays in the absence or presence of Ba100 (200 nM) or Ba25 (200 nM) were set up in 30 ml tissue culture flasks and incubated in a humidified 5% CO_2 in air incubator at 37°C.

PHA-stimulated PBMC were harvested at 24, 40 and 48 h and resuspended at $5x10^6$ cells/ml RPMI 1640/10% AB serum. 100 µl cell suspension aliquots were pipetted into Kimble tubes and cells stained with propidium iodide for DNA quantitation by FACS. Cells were first permeabilised with a non-ionic detergent and then treated with propidium iodide and RNAse (DNA-Prep, a Coulter Reagent kit).

Single parameter histograms using a FL detector 3 (emission spectrum peaks at 650 nm) was used to quantify DNA content.

12. Measurement of proliferating cell nuclear antigen (PCNA) expression by PHA-stimulated PBMCs

PHA-stimulated PBMC proliferation assays in the absence or presence of Ba100 (200 nM) or Ba25 (200 nM) were set up in 30 ml tissue culture flasks and incubated in a humidified 5% CO_2 in air incubator at 37°C. PHA-stimulated PBMCs were harvested at 48 h and resuspended at $2x10^6$ cells/ml RPMI 1640/10% AB serum. Cells were permeabilised with 20 µg/ml lysolecithin and fixed with 1% paraformaldehyde for 2 min at room temperature. The cells were then harvested, resuspended in 200 µl ice cold HPLC grade methanol and incubated on ice for 10 min. The cells were then harvested, 200 µl 0.1% Nonidet P-40 (NP-40) added and cells incubated on ice for 5 min.

The intracellular nuclei were then stained with anti-PCNA and analysed by FACS. The results are expressed as percentage cells expressing PCNA and as the mean fluorescent intensity (extent of PCNA expression/cell).

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13. Effect of Indomethacin on PHA-induced PBMC proliferation in the absence or presence of Ba25

PBMC ($1x10^5$ cells/well) were cultured in triplicate with PHA (0.00575 mitogenic units); PHA + Ba25 (200 nM) or PHA + Ba25 (200 nM) + indomethacin (5 or 10 µg/ml) in a humidified 5% CO_2 in air incubator at 37°C. PBMC were pre-incubated with Ba25 (200 nM) or Ba 25 + indomethacin for 30 min at 37°C prior to the addition of PHA. At 72 h, 1 µCi [^{3}H] thymidine was added and incorporation measured 18 h later.

14. Effect of α-tocopherol on PHA-induced PBMC proliferation in the absence or presence of Ba25

PBMC ($1x10^5$ cells/well) were cultured in triplicate with PHA (0.00575 mitogenic units), PHA + Ba25 (200 nM) or PHA + Ba25 (200 nM) + α-tocopherol (50 or 100 µg/ml) in a humidified 5% CO_2 in air incubator at 37°C. PBMC were pre-incubated with Ba25 (200 nM) or Ba25 + α-tocopherol for 30 min at 37°C prior to the addition of PHA. At 72 h, 1 µCi [^{3}H] thymidine was added and incorporation measured 18 h later.

15. Analysis of data

The results of lymphocyte proliferation assays were expressed as mean DPM of 6 replicates ± SD or as % suppression of lymphocyte proliferation ± SD.

The % suppression of lymphocyte proliferation was calculated using the following formula:

$$\% \text{ Suppression} = \frac{\text{Baseline - Test}}{\text{Baseline}} \times 100$$

Where baseline is the mean DPM of 6 replicates of the control lymphocyte proliferation assay and test is the mean DPM of 6 replicates of lymphocyte proliferation in the presence of Ba100 or Ba25.

We used curve estimation to find the best equation (simple linear, linear with logarithmic transformation, exponential etc.) to analyse the relationship between the concentration of the 2 novel C-type lectins, Ba100 and Ba25 and % suppression of lymphocyte proliferation and to calculate the IC50. Repeated measures analysis was used to compare the % suppression of lymphocyte proliferation at successive concentrations of Ba100 and Ba25 in dose response experiments. The size of the regression coefficient was used to assess the degree of change in % suppression of lymphocyte proliferation with different pre-incubation periods of Ba100 or Ba25 (regression coefficient of 0 is equivalent to no change). The Mann-Whitney test was used to compare small samples where indicated. The Statistical Package for Social Sciences was used for these analyses. Where individual differences were complex, interpretative analysis was done.

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APPENDIX TWO:

ABBREVIATIONS

[^{3}H] thymidine : Tritiated thymidine ^{3}[H] incorporation

ADCC : Antibody-determined cell mediated cytotoxicity

ADP : Adenosine diphosphate

AICD : Activation-induced cell death

Akt : alternative name for PKB

AP-1 : Activator protein 1

APCs : Antigen presenting cells

APTT : Activated partial thromboplastin time

ATG : Antithymocyte globulin

BSA : Bovine serum albumin

CD : Cluster of differentiation

CDK-cyclin : Cyclin dependent kinase-cyclin

CLEC-2 : C-type lectin receptor

CNBr : Cyanogen bromide

Con A : Concanavalin A

COX-1 : Cyclooxygenase enzyme-1

COX-2 : Cyclooxygenase enzyme-2

CRD : Carbohydrate recognition domain

CRE : cAMP-responsive element

CREB/ATF : cAMP-responsive element binding factor

CS-1 : Connecting segment-1

CTLD : C-type lectin-like domain

CTLs : Cytotoxic T cells

d RVVT : dilute Russel's viper venom time

DAG : Diacylglycerol

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DCIR: Dendritic cell inhibitory immunoreceptor

DC-SIGN : Dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (CD209)

dl : Decilitre

DLEC : Dendritic cell lectin

DTH : Delayed-type hypersensitivity

ECD : Glu-Cys-Asp

ECM : Extracellular matrix

eIF-4E complex : eukaryotic initiation factor-4E complex

ELISA : Enzyme linked immunosorbent assay

ERK : Extracellular signal-regulated kinase

FACS : Fluorescence-activated cell sorter

FasL : Fas ligand

FGF : Fibroblast growth factor

FITC-1 : Fluorescein isothiocyanate-1

FKBP : FK506 binding protein

Fn : Fibronectin

FOXP3 : Forkhead/winged helix transcription factor 3

GATA-3 : Member of GATA family of zinc finger proteins

GITR : glucocorticoid-induced TNF-receptor family related receptor

GM-CSF : Granulocyte macrophage-colony stimulating factor

GP : Glycoprotein

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h : Hour

HATT : Heparin-associated thrombocytopaenia and thrombosis

HETE : Hydroxylicosate traenoic acid

HLA : Human leukocyte antigen

HPLC : High performance liquid chromatography

HVEM : Herpes virus entry mediator

ICAM : Intracellular adhesion molecule

IC50 : 50% Inhibitory concentration

ICOS : Inducible costimulator

ICOS-L : Inducible costimulator ligand

IDO : Indole amine 2,3-dioxygenase

IFN-γ : Interferon gamma

Ig : Immunoglobulin

IL : Interleukin

IP-10 : IFN-γ inducible protein

IP3 : Inositol triphosphate

I-TAC : Chemokine CXCL11

ITAM : Immunoreceptor tyrosine-based activation motif

IU : International units

JAK 3 : Janus kinase 3

JNK : N-terminal c-Jun kinase

JT : Jarastatin

KCT : Kaolin clotting time

kDa : Kilodaltons

KGD : Lys-Gly-Asp

KIRs : Killer Ig-like receptors

KTS : Lys-Thr-Ser

L : Ligand

LDV : Leu-Asp-Val

LFA-1 (CD11a/CD18) : Lymphocyte function-associated antigen 1

LFA-3 (CD53) : Lymphocyte function-associated antigen 3

LIBS : Ligand-induced binding site

LIGHT : TNF superfamily member 14 (TNFSF14)

LPS : Lipopolysaccharide

MAPK : Mitogen-activated protein kinase

MCP-1 : Monocyte chemoattractant protein 1

MDG : Met-Asp-Gly

MFI : Mean fluorescent intensity

mg : Milligram

MHC : Major histocompatibility complex

MIG : Monokine-induced by IFN-γ

min : Minutes

MIP-1 : Macrophage inflammatory protein-1

ml : Millilitre

MLD : Met-Leu-Asp

MLR : Mixed lymphocyte reaction

mLT : Membrane lymphotoxin

mRNA : messenger RNA

mTOR : Mammalian target of rapamycin

MVD : Met-Val-Asp

n : Number in study, group

nAChR : Nicotinic acetylcholine receptors

NF-κB : Nuclear factor-κB

NFAT : Nuclear factor of activated T cells

NK cell : Natural killer cell

nM : Nanomolar

NO : Nitric oxide

NP-40 : Nonidet P-40

Oct-1 : Octamer-binding transcription factor

OKT3 : Anti-CD3 antibody

PBMC : Peripheral blood mononuclear cell

PBS : Phosphate buffered saline

PCD : Passive cell death

PCNA : Proliferating cell nuclear antigen

PD-1 : Programmed death-1

PDGF : Platelet derived growth factor

PD-L1 : Programmed death ligand 1

PD-L2 : Programmed death ligand 2

PECAM-1 : Platelet-endothelial cell adhesion molecule

pg : Picogram

PG : Prostaglandin

PGE_2 : Prostaglandin E_2

PHA : Phytohaemagglutinin A

PHAS-I : Phosphorylatable heat stable protein 1 (eIF4E-binding protein)

PI-3 kinase : Phosphotidyl-inositol 3-kinase

PICT : Prothrombinase induced clotting time

PKB : Protein kinase B

PKC : Protein kinase C

PLC : Phospholipase C

PMA : Phorbol 12-myristrate 13-acetate

PPD : Purified protein derivative

PPIases : Peptidyl-prolyl isomerases

PSGL : P-selectin glycoprotein ligand

pSMAC : Peripheral supraclavicular activation cluster

PT : Prothrombin time

PTT : Partial prothrombin time

R-PE : R-Phycoerythrin-conjugated

RANTES : Regulated upon activation normal T cell expressed and secreted

RGD : Arg-Gly-Asp

RGDX : Arg-Gly-Asp (x = Trp, Tyr, Phe, Leu, Val, Cys, Gln or Ser)

RVV-V : Russell's viper venom containing an activator of factor V

RVV-X : Russell's viper venom containing an activator of factor X

SAC : *Staphylococcus aureas* Cowan strain 1

SD : Standard deviation

SDS-PAGE : Sodium dodecyl sulphate polyacrylamide gel electrophoresis

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SE : Standard error

SPSS : Statistical Package for Social Sciences

SRCL : Scavenger receptor C-type lectin

SVMPs : Snake venom metalloproteinases

SVTLEs : Snake venom thrombin-like enzymes

TAP : Transporters associated with antigen processing

T-bet : T-box expressed in T cells

TCR : T cell receptor

TEG : Thromboelastogram

TGF-β : Transforming growth factor-β

Th : T helper cell

TNF : Tumour necrosis factor

TNFR : Tumour necrosis factor receptor

Treg : Regulatory T cell

TTK : Threonine tyrosine kinase

VCAM-1 : Vascular cell adhesion molecule 1

VEGF : Vascular endothelial growth factor

VGD : Val-Gly-Asp

VLA-4 ($\alpha_4\beta_1$) : Very late antigen-4

VLA-5 ($\alpha_5\beta_1$) : Very late antigen-5

vWF : von Willebrand Factor

µl : Microlitre

µg : Microgram