The Effects of *Pseudomonas aeruginosa* Quorum Sensing Signalling Molecules on Human T cell Function

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Abstract

Quorum sensing signalling molecules (QSSMs) are important to *Pseudomonas aeruginosa* virulence and biofilm development which aid establishment and persistence of these bacteria in the host. Recent progress in quorum sensing (QS) research has demonstrated that the two QSSMs, 3-oxo-C12-HSL and PQS interact with eukaryotic cells and modulate immune responses. Early research has indicated these two QSSMs are immunosuppressive, and because T cells play an important role in defending the host against the attack of *P. aeruginosa* (Stevenson *et al.*, 1995), this warrants investigations into the interactions between QSSMs and T cells.

Previous studies have shown 3-oxo-C12-HSL and PQS can exert differential immune-modulatory effects on mammalian immune responses, however, no studies have confirmed these activities using pure human T cells. The purpose of my PhD was to investigate for the first time, the effects of these two QSSMs on pure human T cells in a staged manner, beginning with mouse splenocytes, human peripheral blood mononuclear cells (hPBMCs) and finally pure T cells, then if successful, paving the way for gene array technology.

This present work confirms inhibitory effects by QSSMs on mouse splenocytes stimulated to proliferate using the lectin concanavalin (ConA) or anti-CD3 antibody, and hPBMCs stimulated to proliferate using anti-CD3 and anti-CD28 antibody. In order to further understand interactions between QSSMs and the immune system, the effects of 3-oxo-C12-HSL and PQS on pure human T cell proliferation and cytokine production following stimulation of T cells with monoclonal antibodies directed

against CD3 and CD28 were compared, using CsA as the positive control. All three compounds inhibited pure T cell proliferation. CsA and PQS were the more potent anti-proliferative compounds with IC_{50} values of 3.2±0.31 μM and 3.8±0.15 μM respectively compared to 19±1.62 μM for 3-oxo-C12-HSL, indicating the QSSMs ability to suppress T cell activity and therefore advantageous to *P. aeruginosa*.

To further comprehend the mechanism of action of these two QSSMs, the effects of QSSMs on cytokine production were assessed. 3-oxo-C12-HSL significantly inhibited IL-2 release while PQS enhanced the production of IL-2 even though suppression of T cell proliferation was observed, suggesting a cytostatic effect and demonstrating PQS may in fact act proximally to the IL-2 receptor (IL-2R) or downstream of the T cell signalling pathway, whereas 3-oxo-C12-HSL acts on early T cell signalling events. 3-oxo-C12-HSL also inhibited production of IFNγ in pure T cells and although results were inconclusive for PQS in pure T cell assays, both QSSMs were shown to have an inhibitory effect on IFNγ in mouse splenocytes, suggesting suppression of T cell proliferation is via Th1. Furthermore, 3-oxo-C12-HSL suppressed IL-4, IL-5, IL-10 and TNFα while PQS suppressed IL-10 release at 3.12 μM and enhanced TNFα release, indicating these QSSMs may inhibit T cell proliferation by eliminating both Th1 and Th2 response.

The immune suppressive properties of 3-oxo-C12-HSL and PQS show potential as future therapeutic entities. Immunosuppressive drugs such as CsA and rapamycin are routinely used to maintain transplants and treat auto-immune disorders. However, they can be non-selective and are limited by their side effects including nephrotoxicity and neurotoxicity. Despite recent developments of new

immunosuppressants, there remains an unmet need for less toxic and more widely applicable immunosuppressive agents. 3-oxo-C12-HSL and PQS are worthy of attention as possible future immunosuppressive agents used in conjunction with or in place of present immunosuppressants.

In summary, this study clearly demonstrates for the first time that the two structurally diverse QSSMs, 3-oxo-C12-HSL and PQS, can exert differential modulatory effects on pure T cells, opening a path for further study into their mode of actions within the T cell signalling pathways and their effects at an RNA level.

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List of Abbreviations

2-ME	2-Mercaptoethanol
3-oxo-C12-HSL	*N*-(3-oxododecanoyl) homoserine lactone
AHL	N-acyl homoserine lactone
APCs	Antigen presenting cells
aRNA	anti-sense ribonucleic acid
BSA	Bovine serum albumin
C4-HSL	*N*-butyryl-L-homoserine lactone
CBA	Cytometric bead array
CD	Cluster of differentiation
cDNA	Copy deoxyribonucleic acid
ConA	Concanavalin A
cpm	Count per minute
CsA	Cyclosporin A
CTCM	Complete tissue culture medium
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
EDTA	Ethylenediamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
Erk	Extracellular signal-regulated kinase
EtOH	Ethanol
FACS	Flow cytometry analysis
FCS	Fetal calf serum

GDP	Guanosine diphosphate
GTP	Guanosine 5-triphosphate
IL	Interleukin
IFN	Interferon
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAP	Mitogen-activated protein
MAPK	Mitogen-activated protein kinase
MHC	Major Histocompatibility complex
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethyoxyphenyl)-2-(4-sulfopheyl)-2H-tetrazolium
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PBA	PBS + 1% BSA
PCR	Polymerase chain reaction
PI3-kinase	Phosphoinositide 3-kinase
PPARs	Peroxysomes proliferator activated receptors
PQS	*Pseudomonas* quinolone signal
PTK	Protein tyrosine kinase
QS	Quorum sensing
QSSM	Quorum sensing signalling molecule
RNA	Ribonucleic acid
RNase	Ribonuclease
RPMI	Roswell Park Memorial Institute

RT-PCR	Reverse transcriptase polymerase chain reaction
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
Tregs	Regulatory T cells

1. Introduction

The ultimate purpose of this study is to investigate for the first time the effect of two *P. aeruginosa* quorum sensing signalling molecules (QSSMs) – 3-oxo-C12-HSL and PQS – on the function of purified human T cells. The introduction will include information on the human immune system and more specifically for this research, T cell subsets and their signalling pathways. This introduction will also cover *P. aeruginosa* infections and the purpose of its quorum sensing systems and QSSMs, and how they have been shown to interact with eukaryotic cells.

1.1. The human immune system

The immune system is composed of many interdependent cell types that collectively protect the body from bacterial, parasitic, fungal and viral infections, and from the growth of tumour cells. The immune system protects organisms from infection with layered defences of increasing specificity. Most simply, physical barriers prevent pathogens from entering the body. If these barriers are breached, the innate immune system provides an immediate, but non-specific response. If the innate immune system is evaded by the pathogen, the innate response activates the adaptive immune system, to provide a more specific means of defence and increased protection against subsequent re-infection with the same pathogen (Lydyard *et al.*, 2004). Thus, the innate and adaptive immune systems frequently work together.

1.1.1. The innate immune system

Most infectious agent induces inflammatory responses by activating innate immunity. Microorganisms such as bacteria that penetrate the epithelial surfaces of the body for the first time are met immediately by cells and molecules that can mount an innate immune response. Phagocytic macrophages and neutrophils conduct the defence against bacteria by means of surface receptors that are able to recognise and bind common constituents of many bacterial surfaces. A number of cell surface receptors have been identified that not only act as a first line of defence against many infectious organisms, but also are important to the development of an adaptive immune response (Medzhitov and Janeway, 1997). These surface receptors are called pattern recognition receptors and recognise pathogen-associated molecular patterns (PAMPs) generated by microbes. PAMPs are essential for microbial survival and are conserved structures among many pathogens, which allow innate immunity to recognise microorganisms. Among pattern recognition receptors, Toll-like receptors (TLRs) were highlighted as the key recognition structures of the innate immune system (Akira *et al.*, 2006).

Lipopolysaccharide (LPS) is a cell wall component of gram-negative bacteria. LPS is composed of lipid A, which is recognised by TLR4. LPS recognition by TLR4 requires the formation of a protein complex containing accessory molecules: LPS is generally bound to LPS-binding protein present in the serum, this complex is firstly recognised by CD14 receptor, strongly expressed in peripheral blood monocytes and macrophages (Shimazu *et al.*, 1999, Correia *et al.*, 2001). Once bound to CD14, LPS

comes in close proximity with TLR4 to trigger an inflammatory response (Correia *et al.*, 2001).

Macrophages encountering bacteria in the tissues are triggered to release cytokines that increase the permeability of blood vessels, allowing fluid and proteins to pass into the tissues. Chemokines are also released to direct the migration of neutrophils and monocytes to the site of infection to aid microbial killing. The release of cytokines and chemokines initiate the process of inflammation. Inflammation and the phagocytosis of invading bacteria may also be triggered as a result of the action of complement on the bacterial cell surface. Complement is a system of plasma proteins that activates a cascade of proteolytic reactions on microbial surfaces, coating these surfaces with fragments that are recognised and bound by phagocytic receptors on macrophages (Janeway *et al.*, 2001, Alberts *et al.*, 1994).

Other cells of the innate immune system include natural killer cells, mast cells and basophils. Natural killer cells are important for the protection against viruses and some tumours. Changes in the surface molecules of cells as the result of virus infection allow natural killer cells to bind to and kill infected cells by releasing perforins and inducing apoptosis. In addition, on binding to virus-infected cells, natural killer cells secrete interferon-gamma (IFNγ) which protects adjacent cells from infection by viruses and helps to activate T cell mediated immunity. Mast cells and basophils, when activated, degranulate, releasing pharmacological mediators that cause vasodilation, increased vascular permeability and leukocyte migration.

Also recruited at the site of infection are immature dendritic cells. Dendritic cells (DCs) are important as they represent a primary interface between the innate and adaptive immune systems. Dendritic cells also carries pattern recognition receptors on its surface (Akira *et al.*, 2006, Shimazu *et al.*, 1999). As with macrophages and neutrophils, binding of a bacterium to these receptors stimulates the dendritic cell to engulf the pathogen and degrade it intracellularly. Immature dendritic cells are also continually taking up extracellular material, including any pathogens that maybe present, by the receptor-independent mechanism of macropinocytosis.

The function of dendritic cells, however, is not only primarily to destroy pathogens but also to carry pathogen antigens to peripheral lymphoid organs, and there, present them to T lymphocytes. Immature dendritic cells have immature phenotype that is associated with low levels of major histocompatibility complex (MHC) proteins, and they lack co-stimulatory B7 molecules. Activation of TLRs on DCs by binding to PAMPs on invading microbes forms an important bridge between innate and adaptive immunity. On activation, the dendritic cells matures into highly effective antigen presenting cells (APCs), and through TLRs and antigen presentation, regulates the expression of co-stimulatory molecules on APCs to drive T cell activation, by creating a cytokine milieu to conduct the differentiation of T cells into the desired subset (Re *et al.*, 2001). TLR4 and TLR9 are generally thought to induce a Th1 response by driving IL-12 production by DCs (Krieg, 2002, Trinchieri, 2003), while TLR2 activation might induce a Th2-biased immune response, through production of IL-10 and IL-13 (Dillon *et al.*, 2004).

1.1.2. The acquired immune system

Activated dendritic cells synthesise new MHC class I and II molecules that present peptides of the pathogens at a high level, and express B7 molecules which can co-ordinate naïve T cells and large numbers of adhesion molecules (ICAM-1, ICAM-2, LFA-1 and LFA-3) which enable them to interact with antigen-specific T cells. In addition, activated dendritic cells secrete cytokines that influence both innate and adaptive immune response, making these cell essential gatekeepers that determine whether and how the immune system responds to the presence of infectious agents (Alberts *et al.*, 1994, Janeway *et al.*, 2001).

MHC class I and MHC class II molecules have a distinct distribution among cells that reflects the different effector functions of the T cells that recognise them. MHC class I molecules present peptides from pathogens, commonly viruses, to CD8 cytotoxic T cells, which are specialised to kill cell that they specifically recognise. As viruses can infect any nucleated cell, almost all such cells express MHC class I molecules, although the level of constitutive expression varies from one cell type to the next, e.g. cells of the immune system express abundant MHC class I on their surface, whereas hepatocytes express relatively low levels. In contrast, the main function of the CD4 T cells that recognise MHC class II molecules is to activate other effector cells of the immune system. Thus MHC class II molecules are normally found on B lymphocytes, dendritic cells, and macrophages – cells that participate in immune responses – but not on other tissue cells (Janeway *et al.*, 2001, Alberts *et al.*, 1994).

Naïve T cells recirculate through the secondary lymphoid organs looking for appropriately processed antigen. Initial recognition of processed antigen by T cells is via the T cell receptor (TCR). Accessory molecules provide additional linkages between the APC and the T cell to strengthen their cellular association (Fig. 1.1). CD4 binds to the constant region domain of class II MHC molecules thereby strengthening the association of the TCR with peptide-class II MHC molecules. In contrast, CD8 binds to class I MHC molecules to strengthen the association of the TCR with class I MHC molecules. In addition to engagement of these ligand-receptor pairs, additional adhesion molecules, integrins, become engaged (fig. 1.1). These include intercellular adhesion molecules (ICAMs) and lymphocyte function-associated antigens (LFAs) (Alberts *et al.*, 1994, Janeway *et al.*, 2001).

Ligation of the TCR on its own does not stimulate T cell clonal expression or lymphokine production. The full activation of antigen-specific T cells requires two signals. A first signal, the cluster of differentiation 3 (CD3) antigen is provided through the TCR that interacts with MHC molecules on the membrane of APCs, and signal two is provided by engagement of a co-stimulatory molecule. One of the best characterised co-stimulatory molecules expressed by T cells is CD28, which interacts with CD80 and CD86 on the membrane of APC (fig. 1.1) (AlberolaIla *et al.*, 1997). Co-stimulation allows T cells to become functionally activated, and the outcomes are proliferation and generation of effector populations. CD28 is one important co-receptor that regulates T cell proliferation and survival. T cell activation that culminates in cytokine production, cellular proliferation, and the acquisition of effector functions is initiated by the combination of intracellular

Figure 1.1
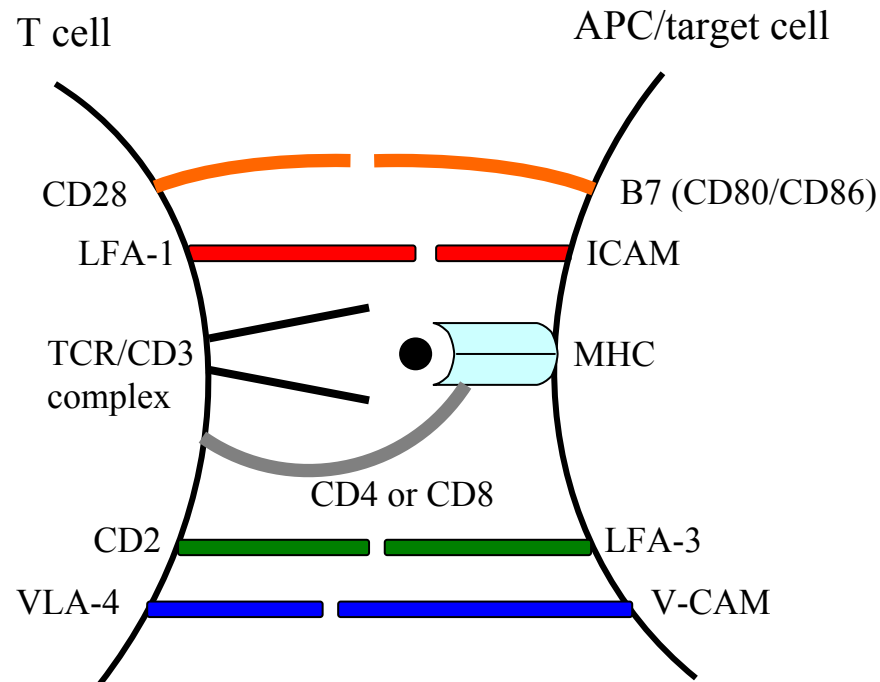
Figure 1.1. Pairs of molecules that strengthen the association of T cells with antigen presenting and target cells. Dendritic cells in lymphoid tissue express high levels of MHC class I and class II molecules, as well as high levels of the adhesion molecules ICAM-1, V-CAM and LFA-3 for efficient T cell binding. Engagement of the TCR/CD3 complex are involved in T cell activation and signal transduction (Clevers *et al.*, 1988), and engagement of the CD28 receptor leads to potent enhancement of lymphokine production (Thompson *et al.*, 1989). Co-stimulation of CD3 and CD28 with anti-CD3 and anti-CD28 leads to complete activation of T cells and a dramatic up-regulation of IL-2 expression (Fraser *et al.*, 1991, Lindsten *et al.*, 1989).

signals emanating from the TCR/CD3 complex and from co-stimulatory molecules such as CD28 (Cantrell, 2002) .

Precursors of CD8^{+} cytotoxic T cells also need to be activated to develop into mature CD8 effector T cells containing granzymes and perforin. This requires attachment of their TCR to MHC class I-peptide complexes on APCs (signal 1). In addition, a second co-stimulatory signal involving binding of B7 to CD28 on the cytotoxic T lymphocyte (CTL) is required. This activation step also induces the expression of FasL on the cytotoxic T cell, which can interact with Fas expressed on the surface of the virus infected cell (Janeway *et al.*, 2001, Alberts *et al.*, 1994, Lydyard *et al.*, 2004).

B lymphocytes are also involved in adaptive immunity. B cells develop from hemopoietic stem cells primarily in the bone marrow. The two main functions of the bone marrow as a primary lymphoid organ are to (a) produce large numbers of B cells, each with unique antigen receptors such that, overall, there is sufficient B cell diversity to recognise the entire antigen are our environment; (b) eliminate B cells with antigen receptors for self molecules. Mature B cells migrate via the bloodstream to the secondary lymphoid organs/tissues. B cells make and use antibodies as their specific antigen receptor. They have molecules similar to CD3 of T cells, i.e. CD79, which are important in their activation, and B cells can mature into plasma cells that produce and secrete large amounts of antibody (Alberts *et al.*, 1994).

Activation of Th cells results in expression of CD40 ligand, the ligand for B cell surface molecule CD40. Th cells now triggers the activation of the B cell via the CD40 surface receptor and as a result, activated B cells reciprocally co-stimulates the Th cell via CD28. Production of IL-4 and IL-5 from Th2 cells act as growth and differentiation factors for B cells promoting IgE and IgA production. IgE plays a significant role in enhancing acute inflammation, in protection from infection by worms, and in allergic reactions (Erb, 2007, Gould *et al.*, 2003). After stimulation of the development of IgE-producing plasma cells by antigen, the IgE produced binds to receptors on mast cells. When antigen is reintroduced into an individual, it binds to the antigen-binding site of the IgE molecule on the mast cell, and as a result of this interaction, the mast cell is triggered to release pharmacologically active agents e.g. histamine (Gould *et al.*, 2003). IgA is synthesised locally by plasma cells in mammary and salivary glands, and along the respiratory, gastrointestinal and genitourinary tracts. IgA is then transported through epithelial cells to the lumen. This antibody is the first line of defence against microbial invaders at mucosal surfaces and in the blood, interacts with immune effector cells to initiate inflammatory reactions. IgA causes antibody-dependent cell-mediated cytotoxicity, degranulation of eosinophils and basophils, phagocytosis by monocytes, macrophages, neutrophils and eosinophils, and triggering of respiratory burst activity by polymorphonuclear leukocytes (Snoeck *et al.*, 2006, Kaetzel *et al.*, 1991).

In addition, IFNγ and TNFα from Th1 cells help promote the development of B cells that produce primarily IgG antibodies, which provides the bulk of immunity to most blood borne infectious agents. IgG can bind to many kinds of pathogens e.g. viruses, bacteria, and fungi, and protects the body against them by complement activation,

opsonisation for phagocytosis and neutralisation of their toxins (Alberts *et al.*, 1994,

Janeway *et al.*, 2001, Lydyard *et al.*, 2004).

1.1.3.   T cells and their subsets

There are two classes of T lymphocytes, cytotoxic T cells and T helper (Th) cells.

Mature cytotoxic T cells, generated with the help of Th1 cells, contain the cytotoxic

machinery required to kill virus-infected cells. These cytotoxic T cells are able to

induce apoptosis of the virus-infected cells through two distinct pathways:

a. Release of lytic granules containing perforin that polymerise in the membrane of

infected cell creating pores that allow entry of granzymes. These enzymes cleave

cellular proteins, the products that initiate induction of apoptosis (Alberts *et al.*,

1994, Janeway *et al.*, 2001).

b. Fas-mediated apoptosis. Viruses' up-regulate expression of Fas (CD95) in

nucleated cells. Cytotoxic T cells activated to release granules by their first

encounter with antigen presented by MHC class I molecules are induced to up-

regulate FasL, which then also allows them to kill specific virus-infected cells by an

additional mechanism through interaction with surface CD95 (Janeway *et al.*, 2001).

The second T cell subset involves production of cytokines and their receptors

following activation of Th cells. These are involved in the expansion and further

differentiation of the T cells into memory and effector cells. On stimulation, T cells

produce interleukin 2 (IL-2), an autocrine growth factor important to T cell proliferation, and express IL-2 receptors (IL-2R). Other surface molecules induced by activation of T cells include CD40L, which interacts with CD40 on dendritic cells. Binding of CD40 induces dendritic cells to produce cytokines required for T cell proliferation and differentiation (Janeway *et al.*, 2001).

Helper T (Th) cells have two important functions: to stimulate cellular immunity and inflammation and to stimulate B cells to produce antibodies. Two functionally distinct subsets of Th cells secrete cytokines that promote these different activities. Th1 cells predominantly produce IL-2, interferon gamma (IFNγ) and tumour necrosis beta (TNFβ) which activates cytotoxic T cells and macrophages to stimulate cellular immunity and inflammation. Th1 cells also secrete IL-3 and GM-CSF (granulocyte macrophage-colony stimulating factor) to stimulate the bone marrow to produce more leukocytes. Th2 cells predominantly secrete IL-4, IL-5, IL-6 and IL-10 which stimulate antibody production by B cells (Janeway *et al.*, 2001).

T cells are initially activated as Th0 cells, which produce IL-2, IL-4 and IFNγ. The nearby cytokine environment then influences differentiation into Th1 or Th2 cells. IL-4 stimulates Th2 activity via the signal transducer and activator of transcription 6 (STAT6) and suppresses Th1 activity, while IL-12 promotes Th1 activities through STAT1. Th1 and Th2 cytokines are antagonistic in activity. Th1 cytokine IFNγ inhibits proliferation of Th2 cells, while Th2 cytokine IL-10 inhibits Th1 secretion of IFNγ and IL-2. Even though there are a number of caveats concerning the differentiation of type 1 versus type 2 Th lymphocytes, one of the most critical elements in determining Th differentiation is the cytokine milieu in which the

T lymphocyte is activated (Borish and Rosenwasser, 1996). Thus, the balance between Th1 and Th2 activity may steer the immune response in the direction of cell-mediated or humoral immunity (fig. 1.2).

Th1/Th2 regulation is exceedingly complex, but its importance is unquestionable, particularly in the study of such diverse disease as allergies and asthma, as well as type 1 diabetes and other autoimmune disorders. For example, rheumatoid arthritis is a dysregulation in Th1/Th2 cell balance, as defined by the production of their specific cytokines, IFNγ and IL-4, respectively, is suggested, leading to eventual destruction of joint tissues. Recently, it has been realized that Th1/Th2 cytokine production offer the unique possibility to predict drug efficacy. For example, IL-5 plays an essential role in orchestrating the eosinophilic inflammation of asthma (Greenfeder *et al.*, 2001). Blocking antibodies to IL-5 inhibit eosinophilic inflammation and airway hyper responsiveness (AHR) in animal models of asthma, including primates (Egan *et al.*, 1996). Humanised monoclonal antibodies to IL-5 have been developed and a single intravenous infusion of one of these antibodies (mepolizulab) markedly reduces blood eosinophils and prevents eosinophil recruitment into the airways after allergen challenge in patients with mild asthma (Leckie *et al.*, 2000). However, this treatment has no significant effect in the early or late response to allergen challenge or baseline AHR, suggesting that eosinophils may not be of critical importance for these responses in humans (Leckie *et al.*, 2000). In addition, Flood-Page *et al.* demonstrated that anti-IL-5 antibody, while profoundly reducing eosinophils in the circulation (by over 95%), is less effective at reducing eosinophils in bronchial biopsies (by approximately 50%), which may explain

Figure 1.2
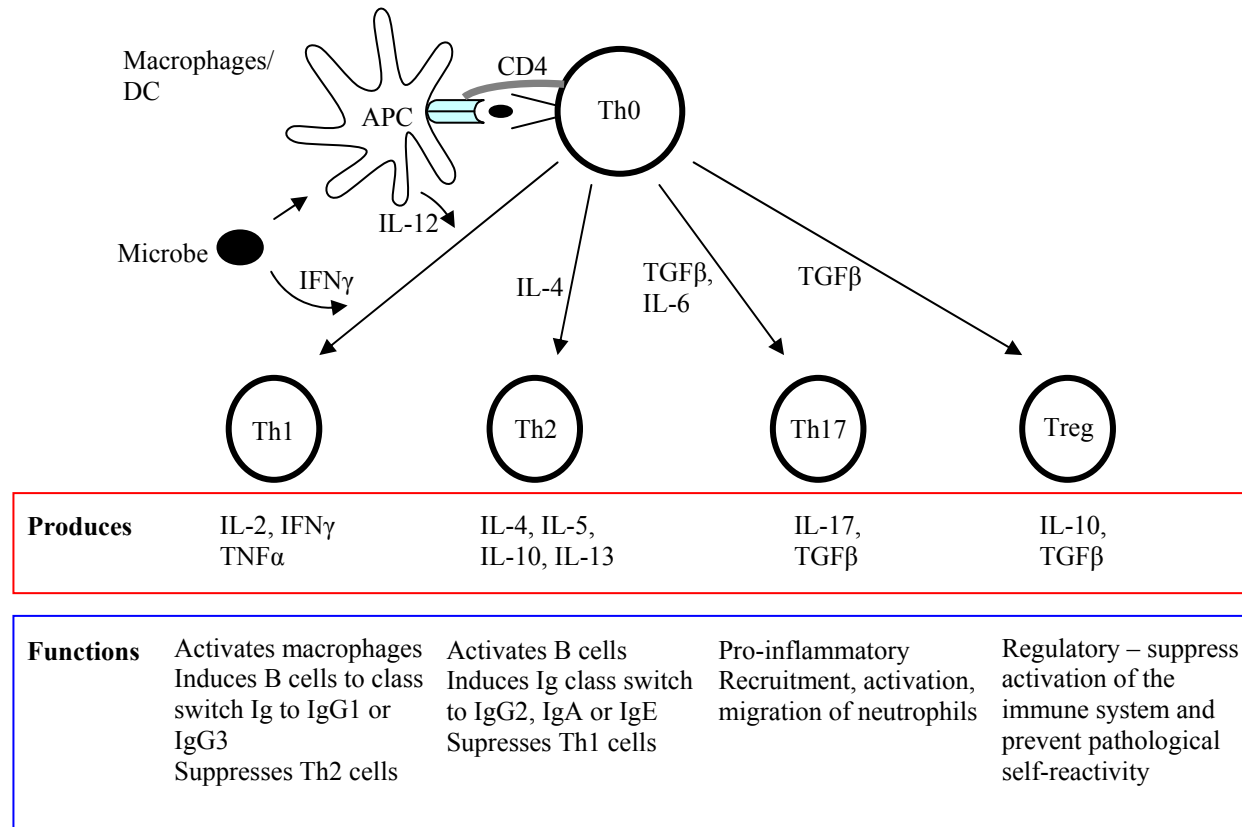
Figure 1.2. Cytokines involved in clonal expansion and development of T cells. Upon antigenic stimulation, naïve CD4$^+$ T cells activate, expand and differentiate into different effector subsets and are characterised by the production of distinct cytokines and effector functions.

why this treatment is not clinically effective (Flood-Page *et al.*, 2003). Nevertheless, this suggests that blocking IL-5 is not likely to be useful approach to asthma therapy.

1.1.4. Th17 lineage

In addition to the Th1/Th2 lineages, recent studies have suggested a greater diversification of the CD4 T cell effector repertoire than that encompassed by the Th1/Th2 paradigm. A new lineage was proposed, characterised by production of members of the IL-17 cytokine group, including IL-17A, B, C, D, IL-17E (also called IL-25) and IL-17F (Kolls and Linden, 2004, Fort *et al.*, 2001) whose development involved mechanisms that are independent of the STAT pathways required for Th1 and Th2 cells (Harrington *et al.*, 2005), and dubbed the 'Th17' lineage. While IL-25 is mainly produced by Th2 cells (Fort *et al.*, 2001) different cell types including T cells, NK cells and neutrophils produce IL-17A and F (Kolls and Linden, 2004). In $CD4^+$ T cells, IL-17A is specifically expressed in a subset of T cells called Th17 for which it became the hallmark cytokine (Langrish *et al.*, 2005) (fig. 1.2).

The majority of studies have focused on the role of IL-17A, IL-17E and IL-17F, with the key function of these three members is to chemoattract different cell types through the induction of other cytokines and chemokines (Kolls and Linden, 2004). IL-25 (IL-17E) induces the expression of Th2-type cytokines and chemokines such as RNATES and Eotaxin-1 and plays a role in Th2-type allergic responses (Fort *et al.*, 2001). IL-17A and IL-17F have pro-inflammatory properties and act on a broad range of cell types to induce the expression of cytokines (IL-6, IL-8, GM-CSF, and

G-CSF) and chemokines (CXCL1, CXCL10). IL-17A and F are also key cytokines for the recruitment, activation and migration of neutrophils (Kolls and Linden, 2004). The production of IL-17 and the recruitment of neutrophils seem important in host protection against gram-negative bacteria and fungal infections. The preferential production of IL-17 by T cells during infections with *Klebsiella pneumonia* (Ye *et al.*, 2001), *Bacteroides fragilis* (Chung *et al.*, 2003), *Mycobacterium tuberculosis* (Infante-Duarte *et al.*, 2000), and fungal species (LeibundGut-Landmann *et al.*, 2007) suggests that Th17 responses are triggered by specific pathogens, probably through TLR4 (Pirhonen *et al.*, 2007, Eijnden *et al.*, 2006) and are required for their clearance. In addition to neutrophils, IL-17 might also dictate the migration of other important effector cell types during infection, for example, pathogen-specific Th17 cells generated during mycobacterial infection induce the expression of CXCL9, CXCL10 and CXCL11, which attract IFNγ-producing CD4$^+$ Th1 cells to the lung in order to control the infection (Khader *et al.*, 2007).

Furthermore, Th17 cells might potentially play an important role in asthma, autoimmune diseases such as rheumatoid arthritis (RA) and allograft rejection. The recruitment of neutrophils which are prominent in acute, severe exacerbations of asthma (Jatakanon *et al.*, 1999), IL-17 is expressed in bronchial biopsies, bronchoalveolar lavage fluid and sputum of patients with asthma (Molet *et al.*, 2001, Sun *et al.*, 2005, Barczyk *et al.*, 2003). Levels of IL-17 are elevated in the synovium of patients with RA (Kotake *et al.*, 1999), and synovial cultures from patients with RA spontaneously secrete IL-17 (Chabaud *et al.*, 1999); the source of this IL-17 is local production by T cells (Chabaud *et al.*, 1999) and juxta-articular bone lymphocytes (Chabaud *et al.*, 2001). Pathologically, this cytokine can activate and

enhance mechanisms involved in RA such as up-regulate and/or synergise with local inflammatory mediator such as IL-6, IL-1β and TNFα (Chabaud *et al.*, 1998, Katz *et al.*, 2001, LeGrand *et al.*, 2001). In human lung organ transplantation, IL-17 has been reported as being elevated during acute rejection (Vanaudenaerde *et al.*, 2006), while rat models have demonstrated that collagen type V-specific lymphocytes can mediate lung allograft rejection and express IL-17 locally at the site of rejection (Yoshida *et al.*, 2006). In cardiac allograft models, antagonism of the IL-17 network (via expression of an IL-17R-immunoglobin fusion protein) can reduce intragraft production of inflammatory cytokines (namely IFNγ) and prolong graft survival (Li *et al.*, 2006).

Recently, it has been recognised that a combination of two cytokines, namely IL-6 and transforming growth factor beta (TGFβ) is necessary for the differentiation of naïve T cells to Th17 cells (Veldhoen *et al.*, 2006, Bettelli *et al.*, 2006, Mangan *et al.*, 2006). Veldhoen *et al.* determined naïve precursor T cells could be skewed towards a Th17 phenotype in the presence of dendritic cells and Tregs in an inflammatory milieu (lipopolysaccharide stimulation) (Veldhoen *et al.*, 2006). In the presence of Tregs and DC, the important drivers of Th17 differentiation were Treg-derived TGFβ and DC-derived IL-6, although both TNFα and IL-1β (both DC-derived) also augmented the commitment to Th17. These data were corroborated by Bettelli *et al.*, who demonstrated using cells from a *Foxp3*-GFP knock-in mice strain that differentiation towards Treg and Th17 phenotypes were mutually exclusive. Activation of naïve precursor cells using anti-CD3 in the presence of TGFβ lead to production of Tregs, but activation in the presence of IL-6 in addition to TGFβ

completely abrogated this and led to development of Th17 cells (Bettelli *et al.*, 2006).

1.1.5. Regulatory T cells

Regulatory T cells are a specialized subpopulation of T cells that act to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self-antigens. Given that both human and murine knock-outs for $CD4^+$ $CD25^+$ T cells (Tregs) develop severe autoimmune diseases (Sakaguchi *et al.*, 1996, Sakaguchi *et al.*, 1995), the focus of attention in the literature has been mainly on these Tregs. Naturally arising $CD4^+$ $CD25^+$ Tregs, which constitute 5-10% of peripheral $CD4^+$ T cells in normal rodents and humans, are produced at least in part by the normal thymus as functionally mature and distinct subpopulation of T cells (Sakaguchi, 2004). *In vitro*, Tregs have the ability to inhibit proliferation and production of cytokines by responder ($CD4^+$ $CD25^-$ and $CD8^+$) T cells (Jonuleit *et al.*, 2001, Dieckmann *et al.*, 2001, Thornton and Shevach, 1998) to polyclonal stimuli, as well as to down-modulate the responses of $CD8^+$ T cells, NK cells and $CD4^+$ cells to specific antigens (Dieckmann *et al.*, 2001, Wing *et al.*, 2003). These predicates translate *in vivo* to a greater number of functions other than the maintenance of tolerance to self-components (i.e. prevention of autoimmune disease) (Baecher-Allan and Hafler, 2006), and include control of allergic diseases (Umetsu and DeKruyff, 2006) and regulation of responses to microbial pathogens (Rouse *et al.*, 2006), as well as the ability to prevent transplant rejection (Waldmann *et al.*, 2006).

The latest research suggests that Tregs are defined by expression of the forkhead family transcription factor *Foxp3* (Fontenot *et al.*, 2005, Sakaguchi, 2005). The large majority of *Foxp3*-expressing regulatory T cells are found within the MHC class II restricted $CD4^+$ T cell population and express high levels of the IL-2 receptor (IL-2R) alpha chain (CD25) (Fontenot and Rudensky, 2005, Sakaguchi, 2005). Setoguchi *et al.* showed that IL-2 is essential for physiological expansion and survival of natural of natural $CD4^+$ $CD25^+$ Tregs in the periphery, and that neutralisation of IL-2 in normal mice inhibited the physiological proliferation of peripheral $CD4^+$ $CD25^+$ Tregs. IL-2 neutralisation in normal mice for a limited period also induced autoimmune disease similar to the one produced by depletion of $CD4^+$ $CD25^+$ Tregs (Setoguchi *et al.*, 2005). In addition to the *Foxp3*-expressing $CD4^+$ $CD25^+$, there also appears to be a minor population of MHC class I restricted $CD8^+$ *Foxp3*-expressing regulatory T cells (Belkaid and Rouse, 2005).

1.2. Cytokine signalling pathways

Cross-linking of the TCR through the binding of peptide-MHC complexes on the membrane of APC or with antibodies to CD3 initiates cytokine production. Cytokines initiate intracellular signals through the binding and oligomerisation of receptor subunits. The common outcome of each receptor oligomerisation event causes the rapid stimulation of associated protein tyrosine kinases (PTK) such as the JAK and the Src family kinases (AlberolaIla *et al.*, 1997, Cantrell, 2002), this triggers activation of a plethora of downstream signalling molecules (AlberolaIla *et al.*, 1997, Cantrell, 2002). In contrast to JAK kinases that function specifically in cytokine receptor signalling, Src kinases has been implicated in numerous cellular

processes. However, several members of the Src family including Src, Lck, Fyn and Lyn have also been implicated in cytokine signalling (Miyajima *et al.*, 1992, AlberolaIla *et al.*, 1997, Cantrell, 2002). Cellular responses induced by cytokine stimulation may be categorised into several general types – cellular proliferation, activation of specific genes and in some cases cell death.

Important components of these intracellular mediators are the Ras/mitogen-activated protein kinases (MAPKs), most often associated with cellular proliferation. The Ras/MAPK pathway was one of the first pathways in which extracellular signals were biochemically and genetically linked to activation of gene expression in the nucleus (Marshall, 1994). The central components of this pathway are a series of related small (21 KDa) GTPases, the best characterised of which are the p21 Ras proteins. Ras proteins undergo post translational modification which localises the Ras proteins to the plasma membrane (Hancock *et al.*, 1990). Membrane associated Ras binds GTP, which is then hydrolysed to GDP by the GTPase activity of Ras. Cytokine stimulation results in a rapid increase in the amount of Ras-GTP, the biologically active form that initiates downstream cascade of kinase reactions termed the MAPK pathway (Hall and Self, 1986). In the Ras/MAPK cascade, two specific isoforms of MAPK kinases (MAPKK) have been identified and are termed MEK1 and MEK2 (MAP/Erk kinases) (Denhardt, 1996). Activation of the MEKs results in the phosphorylation and activation of MAPK. As with MAPKK, two isoforms of MAPK have been identified and are referred to as extracellular signal-regulated kinase 1 and 2 (Erk1/2) (fig. 1.3) (Denhardt, 1996). In turn, these kinases activate specific T cell factors (TCF)/transcription factors and early immune response genes such as the IL-2 and IL-2 receptor (IL-2R) genes (Garrington and Johnson, 1999).

Figure 1.3
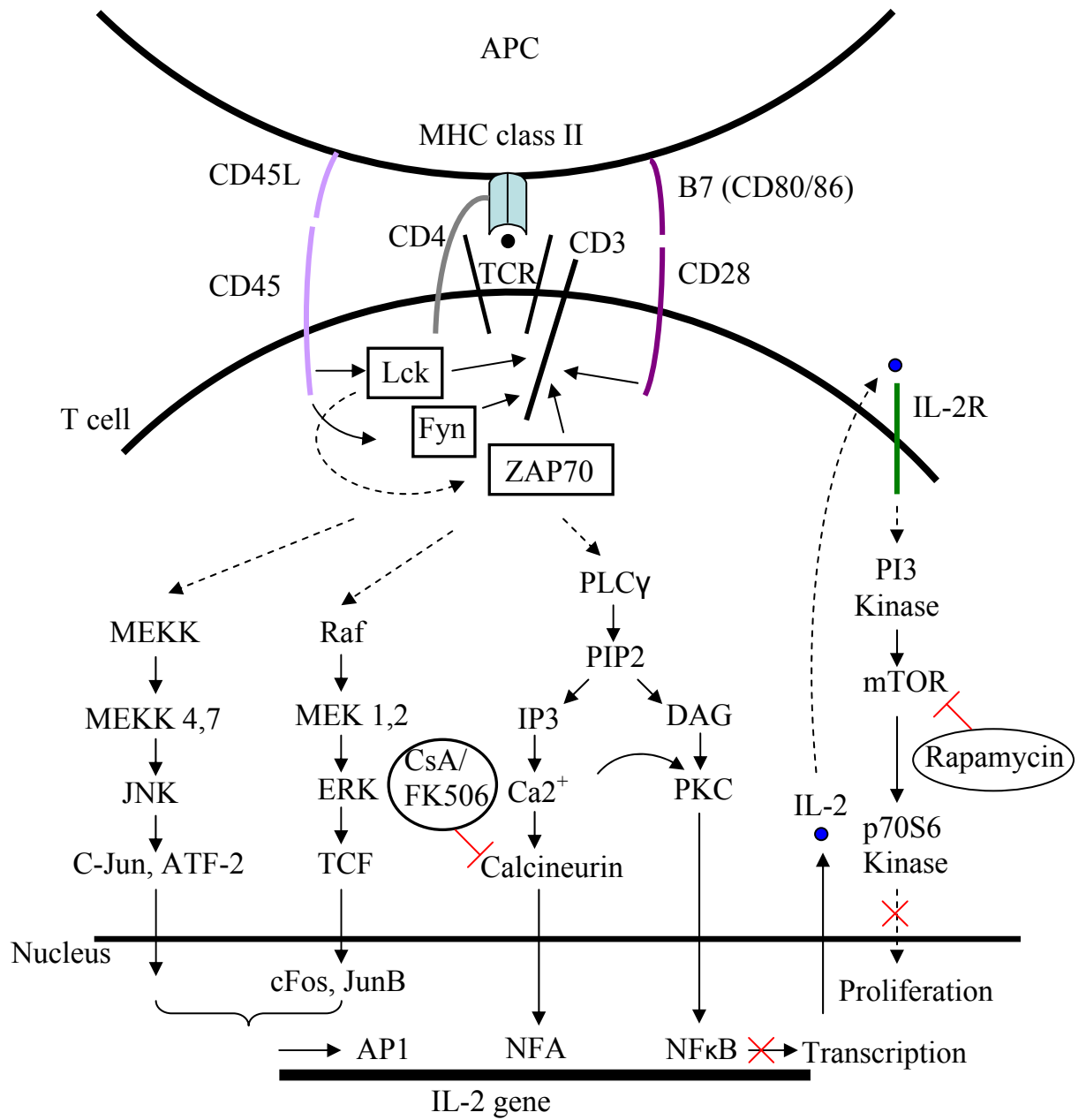
Figure 1.3. Early biochemical events leading to T cell activation. Activation of cytokines leads to initiation of intracellular signals through the binding and oligomerisation of receptor subunits. The outcome of this is the activation of tyrosine kinases followed by phosphorylation of cellular substrates. Cytokine activation of the Ras/MAPK is most often associated with cellular proliferation. Activation of PI-3 kinase is also clearly important in the stimulation of cellular proliferation by numerous cytokines including IL-2. Currently employed immunosuppressive drugs include cyclosporin A (CsA), FK506 and rapamycin. CsA and FK506 inhibit calcineurin, preventing the activation of NFAT. Rapamycin inhibits the serine-threonine kinase mTOR, the key regulator of translation.

ERKs in particular catalyse the phosphorylation of the transcription factor Elk-1, resulting in the activation of the cFos proto-oncogene (Davis, 1995). Disruption of the MAPK kinase (MEK) 1/Erk pathway with specific inhibitors suppresses the proliferative response and the production of several cytokines by T cells (fig. 1.3) (Dumont *et al.*, 1998).

Distinct kinases with functions similar to the ERKs can be activated through kinase cascades initiated by Ras as well as other small GTPases. These kinases include Jun nuclear kinases (JNK, which phosphorylate the transcription factor Jun, p38 kinase, which phosphorylates the transcription factor ATF2, and p90rsk, which phosphorylates Fos (Denhardt, 1996, Davis, 1995). Signals leading to the activation of these different transcription factors are often co-ordinated. The activation of the cFos gene by ERK-activated Elk-1 and the activation of Jun by JNK phosphorylation leads to the formation of AP-1, a transcription activator composed of cFos and Jun heterodimers that is involved in the transcription of numerous genes (Davis, 1995). Ultimately, this induction of gene expression leads to many of the cellular responses attributed to the activation of the Ras/MAPK pathway.

Many aspects of the phosphoinositide 3-kinase (PI3-kinase) pathway, a second signalling pathway activated by cytokines, have proven to differ from the Ras/MAPK pathway. The activation of PI3-kinase is clearly important in the stimulation of cellular proliferation by numerous cytokines including IL-2, IL-3, IL-4, IL-5 and GM-CSF (Gold *et al.*, 1994, Merida *et al.*, 1991). PI-3-kinase is composed of a p110 catalytic subunit and a p85 regulatory subunit. The p85 subunit contains an N-terminal SH3 domain and two praline-rich regions that interact with

SH3 domains in other signalling molecules (Kapeller *et al.*, 1994). The C-terminal SH2 domain links the p85/p110 heterodimer to tyrosine phosphorylated proteins and activated receptor molecules, resulting in bringing PI3-kinase to the membrane near activated receptor complexes (Kapeller and Cantley, 1994, Kapeller *et al.*, 1994). Once recruited to activated receptor complexes, PI3-kinase is activated by several mechanism. Direct interaction of the SH2 domains with phosphorylated receptors or adaptor molecules induce conformational change resulting in the increased catalytic activity of the p110 subunit (Shoelson *et al.*, 1993). It is also likely that JAK kinase activation leads to p85 phosphorylation since engagement of different cytokine receptors that associate with JAK kinases induce the tyrosine phosphorylation of the p85 subunit (Ruizlarrea *et al.*, 1993). Activation of PI3-kinase also may occur from its interaction with activated Ras-GTP molecules in a situation analogous to the activation of Raf-1 by Ras-GTP (Rodriguezviciana *et al.*, 1994).

The phosphorylated lipids resulting from the activation of PI3-kinase act as second messengers that activate a number of cellular kinases by interacting directly with these proteins. In particular, $PtdIns(3,4,5)P_3$ and $PtdIns(3,4)P_2$ have been shown to act as potent activators of Ca^{2+}-independent forms of protein kinase C, which phosphorylate and alter the activity of numerous cellular substrates (Toker *et al.*, 1994) (fig. 1.3).

Analysis of cytokine production and the subsequent T cell signalling pathways is increasingly important in defining the course of an immune response and in evaluating specific therapies of immune diseases as well as playing a pivotal role in the immune response to allograft rejection.

1.2.1. The effect of immunosuppressive agents on the T cell signalling pathways

The development of effective and safe immunosuppressive procedures for the treatment of organ transplant rejection and autoimmune allergic and inflammatory diseases represents a major goal in contemporary medicine. Because T cell activation plays a central role in the regulation of immune responses, its pharmacologic inhibition has provided a powerful approach towards this goal. The currently employed immunosuppressive drugs such as CsA, FK506 and rapamycin, which have a degree of selective action on cells of the immune system, has led to a dramatic increase in the success rate of transplant surgery despite their side effects. CsA can be nephrotoxic and rapamycin can cause immune-mediated toxicity (Allison, 2000), indicating the importance of further immunosuppressive products to clinical medicine and also an understanding of molecular mechanisms involved (Allison, 2000).

Rapamycin treatment has been shown to cause Gap 1 (G_1) arrest in a variety of cell types including T cells (Schmelzle and Hall, 2000). In proliferating cells, the cell cycle consists of four phases and the G_1 phase is the interval between mitosis and DNA replication that is characterised by cell growth. The transition that occurs at the restriction point in G_1 commits the cell to the proliferative cycle. If the conditions that signal this transition are not present or blocked, the cell exits the cell cycle and enters G_o, a non-proliferative phase. The inhibition of T cell proliferation is primarily due to the blockage of IL-2 signalling (Powell *et al.*, 1998, Brennan *et al.*, 1999) (fig. 1.3). It has been well established that rapamycin inhibits the

serine-threonine kinase mTOR (mammalian target of rapamycin)/FRAP (FKBP12-rapamycin-associated protein) (Brown *et al.*, 1994, Chiu *et al.*, 1994, Sabatini *et al.*, 1994, Almawi *et al.*, 1999, Gingras *et al.*, 2001) and that mTOR is a key regulator of translation (Gingras *et al.*, 2001) (fig. 1.3).   Two of the most important components in the translational process that are regulated by mTOR are 4EBP-1 and $p70^{s6k}$ (Gingras *et al.*, 2001).   4EBP-1 is a repressor of eukaryotic initiation factor (eIF4E) and the availability of eIF4E is critical for translation, thus rapamycin has been suggested to affect translation of polypyrimidine-tract containing mRNAs (Jefferies *et al.*, 1994).

CsA inhibits T cell growth factor gene expression at the level of mRNA transcription (Kronke *et al.*, 1984).   Liu *et al.* (1991) found that CsA inhibits the phosphatase activity of calcineurin (fig. 1.3).   By preventing their calcineurin-mediated dephosphorylation, CsA inhibits the translocation of the NFAT family of transcription factors from the cytoplasm to the nucleus of activated T cells (Liu *et al.*, 1991).   The NFAT group is involved in the transcriptional activation of the genes encoding IL-2, IL-12 and the CD40L.   In addition, CsA has recently been found to block the JNK and p38 signalling pathways triggered by antigen recognition in T cells (Matsuda and Koyasu, 2000).   The presence of two target pathways for CsA in T cells could explain the high specificity of its immunosuppressive effects.

In addition, as rapamycin and CsA are widely used to prevent allograft rejection, and the development of an alloimmune response into rejection or stable allograft tolerance is strongly determined by the balance between alloreactive effector cells and CD4+CD25$^+$ Tregs (Coenen *et al.*, 2005, Zheng *et al.*, 2003), it remains to be

elucidated whether these drugs have an effect on CD4$^+$CD25+ Tregs. Recently, Qu *et al.* observed that rapamycin significantly enhanced the relative levels of CD4$^+$CD25$^+$ Tregs in the spleen and thymi of mice while most of T cell subsets were decreased. Although rapamycin inhibited the immune response of CD4$^+$ T cells to allogenic antigen or mitogens, the immunosuppressive function of CD4$^+$CD25$^+$ Treg cells was not impacted by the treatment of rapamycin in *in vitro* and *in vivo* functional assays (Qu *et al.*, 2007). This may suggest that the treatment with rapamycin may selectively inhibit the host immune rejection against grafts while still keep the host with the potential ability to induce immune tolerance. This may be beneficial to patients who have transplant grafts or autoimmune diseases, as they have the tendency to achieve immune tolerance. In contrast to rapamycin, Zeiser *et al.* determined that CsA did not abrogate but significantly reduced the function of allostimulated Treg cells *in vitro* (Zeiser *et al.*, 2006). Reduced FoxP3 gene transcription of murine Treg cells when exposed to CsA, but not to rapamycin, is consistent with a recent study showing that calcineurin inhibitors reduced FoxP3 expression in human Treg cells, whereas mTOR inhibitors have little or no impact (Baan *et al.*, 2005).

Furthermore, Coenen *et al.* reported that allogenic expansion of human naturally occurring CD4$^+$CD25$^+$ Tregs leads to the emergence of a distinct, highly suppressive CD27$^+$ Treg subset next to a CD27$^-$ Treg subset which has modest suppressive potency (Coenen *et al.*, 2005, Koenen *et al.*, 2005). The CD27$^+$ Treg subset was shown to suppress not only naïve and antigen-experience memory T cells but also ongoing T cell responses. Coenen *et al.* demonstrated that rapamycin and CsA act differently with regard to the preservation of the high CD27$^+$ Treg/CD27$^-$ Treg

ration. In contrast to CsA, rapamycin preserved the dominance of CD27$^+$ Tregs over CD27$^-$ Tregs on allogenic stimulation, resulting in strong suppressive capacity of the expanded CD4$^+$CD25$^+$ Treg pool as a whole (Coenen *et al.*, 2006). It can therefore be envisaged that a predominance of CD27$^+$ Tregs, which is supported by treatment with rapamycin, can add to the development of stable tolerance and prevention of rejection in solid-organ transplantation.

Cross-linking of CD27 has been shown to induce proliferation following the mobilisation of intracellular free Ca^{2+} (Croft, 2003, Dejong *et al.*, 1991). As CsA specifically targets Ca^{2+}-dependent activation pathways, CsA may thus inhibit CD27$^+$ Treg proliferation, however, preliminary data from Coenen *et al.* do not support an inhibition of proliferation.

It has been proposed that signalling by the growth factor IL-2 is crucial for the functionally activity of Tregs (Malek and Bayer, 2004, Thornton *et al.*, 2004). As mentioned earlier, CsA inhibits TCR-induced activation and IL-2 production, whereas rapamycin blocks signalling in response to T cell growth factors. In theory, both drugs could therefore interfere with Treg function. However recent studies observed that in the presence of CsA or rapamycin freshly isolated CD4$^+$CD25$^+$ Tregs were able to suppress CD4$^+$CD25$^-$ T cells indicating that CsA and rapamycin did not significantly interfere in the activation or suppressor function of these cells (Coenen *et al.*, 2006).

Immunomodulatory small molecules with new modes of action such as the compounds examined in this present study could therefore be extremely useful for

immunology research and crucially, are potential for the treatment of autoimmune diseases such as rheumatoid arthritis and multiple sclerosis, and for the maintenance of transplants.

1.3. *Pseudomonas aeruginosa*

1.3.1. Background on *P. aeruginosa*

Pseudomonas aeruginosa is a gram negative bacterium capable of causing serious infections in insects, plants and animals (McGrath, 2003). *P. aeruginosa* emerged as a human pathogen in the past century, probably because of the development of available niches after the eradication of the "old" pathogens by use of antibiotics and disinfectants. As an opportunistic pathogen of humans, *P. aeruginosa* is a major cause of nosocomial infections and is responsible for the chronic lung infections that affect most cystic fibrosis (CF) patients (Van Delden and Iglewski, 1998, Smith *et al.*, 2001, McGrath, 2003). *P. aeruginosa* pneumonia is also an emerging problem and in many cases is fatal in patients with advanced HIV infections (Meynard *et al.*, 1999, Smith *et al.*, 2001) and ventilator-associated pneumonia (Dunn and Wunderink, 1995, Brewer *et al.*, 1996, Smith *et al.*, 2001).

Once acquired, *P. aeruginosa* infections are difficult to eradicate using current antimicrobial therapies due to its inherent antimicrobial resistance mechanisms (Wagner *et al.*, 2004). The ability of *P. aeruginosa* to colonise a wide variety of anatomical sites and produce such diverse and often overwhelming infections, is due to an arsenal of well-regulated virulence factors which are capable of causing

extensive tissue damage and bloodstream invasion consequently promoting systemic dissemination (Van Delden and Iglewski, 1998, Calfee *et al.*, 2001). Chronic infections such as CF lung infections and colonisation of indwelling medical devices (e.g. catheters), are characterised by biofilm formation, localised containment of the bacteria to a specific area of the body and persistence (Furukawa *et al.*, 2006). During the course of CF, *P. aeruginosa* forms biofilms characterised by cellular aggregates embedded within the mucus layer present in the airways (Lam *et al.*, 1980). After colonisation occurs, antibiotic therapy can never completely eradicate the bacteria even though high levels of antibiotics can be achieved in the airway secretions. Colonisation of urinary tract catheters is another example of a *P. aeruginosa* chronic infection. Stickler *et al.* demonstrated that acyl-homoserine lactone signals were produced by *P. aeruginosa* biofilms growing on the surface of a catheter (Stickler *et al.*, 1998).

1.3.2. Bacterial pathogenicity factors

The failure of the bacterium to infect a healthy individual means the human immune system and more importantly T cells play an essential role in defending the host against *P. aeruginosa*. However, in immunocompromised patients, an array of virulence factors may work to prevent establishment of an immune response and aid maintenance of the bacteria in the host. For example, exotoxin A is toxic to macrophages, is a T cell mitogen and inhibits granulocyte and macrophage progenitor cell proliferation (Pearson *et a*l., 1997). Exotoxin A is also responsible for local tissue damage, bacterial invasion (Woods and Iglewski, 1983), and immunosuppression (Vidal *et al.*, 1993). Purified exotoxin A is highly lethal for a

mouse which supports its role as a major systemic virulence factor of *P. aeruginosa* (Woods and Iglewski, 1983). Exoenzyme S is responsible for direct tissue destruction in lung infections (Nicas *et al.*, 1985b) and may be important for bacterial dissemination (Nicas *et al.*, 1985a). The two hemolysins, phospholipase C and rhamnolipid may act synergistically to break down lipids and lecithin. Both may contribute to tissue invasion by their cytotoxic effects. Rhamnolipid is believed to solubilise the phospholipids of lung surfactant making them more accessible to cleavage by phospholipase C (Liu, 1974) and also inhibits the mucoiliary transport and ciliary function of human respiratory epithelium (Read *et al.*, 1992).

P. aeruginosa also produces several proteases including Las B elastase and LasA elastase. The ability of *P. aeruginosa* to destroy the protein elastin is a major virulence determinant during acute infection. Elastin is a major part of human lung tissue and is responsible for lung expansion and contraction. It is also an important component of blood vessels, which rely on it for their resilience. The concerted activity of LasB elastase and LasA elastase is believed to be responsible for destroying elastin-containing human lung tissue and cause the pulmonary haemorrhages of invasive *P. aeruginosa* infections (Galloway, 1991). LasA nicks elastin, rendering it sensitive to degradation by other proteases such as LasB elastase, alkaline protease and neutrophils elastase (Galloway, 1991). LasB elastin also degrades fibrin and collagen (Heck *et al.*, 1986), can inactivate substances such as human immunoglobulin G and A (Heck *et al.*, 1990), airway lysozyme (Jacquot *et al.*, 1985), complement components (Hong and Ghebrehiwet, 1992), and substances involved in protecting the respiratory tract against proteases such as alpha-1-protease inhibitor and bronchial mucus proteinase inhibitor (Morihara *et al.*, 1979, Johnson *et*

al., 1982).   Therefore, LasB elastase not only destroys tissue components but also interferes with host defence mechanisms.   Studies in animal models demonstrated that mutants defective in LasB elastase production were less virulent than their parent strains (Nicas and Iglewski, 1985, Tamura *et al.*, 1992, Tang *et al.*, 1996), which supports the role of LasB elastase as a virulence factor.   Pyocyanin inhibits lymphocyte proliferation and leukocidin is cytotoxic to neutrophils and lymphocytes (Pearson *et al.*, 1997, Wilson and Dowling, 1998).

Many of these extracellular virulence factors are crucial for the competence of *P. aeruginosa* to establish and maintain the infection and have been shown to be controlled by a complex regulatory circuit involving cell-to-cell signalling systems termed quorum sensing (QS) (Telford *et al.*, 1998, Van Delden and Iglewski, 1998, McGrath, 2003, Smith and Iglewski, 2003a).   Mutants defective in quorum sensing are typically compromised in their ability to establish a successful infection, for example, mutants defective in quorum sensing were substantially less pathogenic than their parental strains in the burned mouse model, the mouse agar bead model or in the neonatal mouse model of pneumonia (Tang *et al.*, 1996, Rumbaugh *et al.*, 1999, Wu *et al.*, 2000).   Thus understanding this QS network and its mode of action within the host may prove important in combating *P. aeruginosa* infections, and its importance in T cells function is further discussed in the next sections.

1.4. *P. aeruginosa* **quorum sensing systems and quorum sensing signalling molecules**

QS is a generic regulatory mechanism that allows bacteria to launch a unified, co-ordinated response in a population density-dependent manner to accomplish tasks that would be difficult, if not impossible to achieve for a single cell. QS systems of gram positive bacteria involve peptide pheromones as signals (Kleerebezem *et al.*, 1997). In contrast, QS systems in gram negative bacteria are composed of a small molecule called an autoinducer with the various types of autoinducers described in gram negative bacteria are homoserine lactose-based molecules (Fuqua *et al.*, 1996).

P. aeruginosa has one of the most extensively studied QS systems. In this organism, QS is complex and enables the regulation of genes by the production of small diffusible N-acyl homoserine lactone signal molecules (AHLs) also termed quorum sensing signal molecules (QSSMs) (Finch *et al*., 1998, Smith *et al.*, 2001). At low cell density, autoinducers are synthesised at basal levels and is thought to diffuse into the surrounding media where it becomes diluted. With increasing cell density, the intracellular concentration of autoinducer increases until it reaches a threshold concentration. At this critical concentration, the autoinducer has been reported to bind to a LuxR-type transcriptional activator protein (R-protein) (Greenberg, 1997). The R-protein/autoinducer complex has been proposed to bind specific DNA sequences upstream of target genes enhancing their transcription (Stevens *et al.*, 1994, Stevens and Greenberg, 1997). QSSMs therefore allow bacteria to express specific genes as a population.

P. aeruginosa produces at least three small compounds that function as intercellular communication signals.   The two AHL signal molecules, *N*-(3-oxododecanoyl) homoserine lactone (3-oxo-C12-HSL) and *N*-butyryl-L-homoserine lactone (C4-HSL), have been well studied and function in combination with the LuxR homologs *LasR* and *RhlR*, respectively (Gambello and Iglewski, 1991, Pearson *et al.*, 1994, Pearson *et al.*, 1995, Calfee *et al.*, 2001, Smith *et al.*, 2001, McGrath, 2003). Together, these QS signals control 4-12% of the *P. aeruginosa* genome (Whiteley *et al.*, 1999, Calfee *et al.*, 2001, McGrath, 2003, Schuster *et al.*, 2003, Wagner *et al.*, 2003).   The third *P. aeruginosa* intercellular signal, 2-heptyl-3-hydroxy-4-quinolone (the *Pseudomonas* quinolone signal [PQS]) (Pesci *et al.*, 1999) controls multiple virulence factors and is intertwined in the QS cascade, where it appears to be a regulatory link between the *las* and *rhl* QS systems (McKnight *et al.*, 2000, Diggle *et al.*, 2003).

To elaborate, in *las* QS , the 3-oxo-C12-HSL synthase, lasI, directs the synthesis of primarily 3-oxo-C12-HSL (Pearson *et al.*, 1994), triggering the las R-encoded transcriptional activator, LasR (fig. 1.4) (Gambello and Iglewski, 1991, Pearson *et al.*, 1997), to induce a number of virulence genes including lasB, lasA, apr, toxA, and lasI itself (Toder *et al.*, 1991, Gambello *et al.*, 1993, Passador *et al.*, 1993). The *las* system also auto-regulates lasI, leading to the production of more 3-oxo-C12-HSL (Seed *et al.*, 1995). In addition, the *las* system controls expression of the *xcpP* and *xcpR* genes that are involved in the regulation of the Type II general secretion pathway (Chapon-Herve *et al.*, 1997) and has been implicated in the maturation of *P. aeruginosa* biofilms (Davies *et al.*, 1998). Although 3-oxo-C12-HSL is diffusible,

Figure 1.4
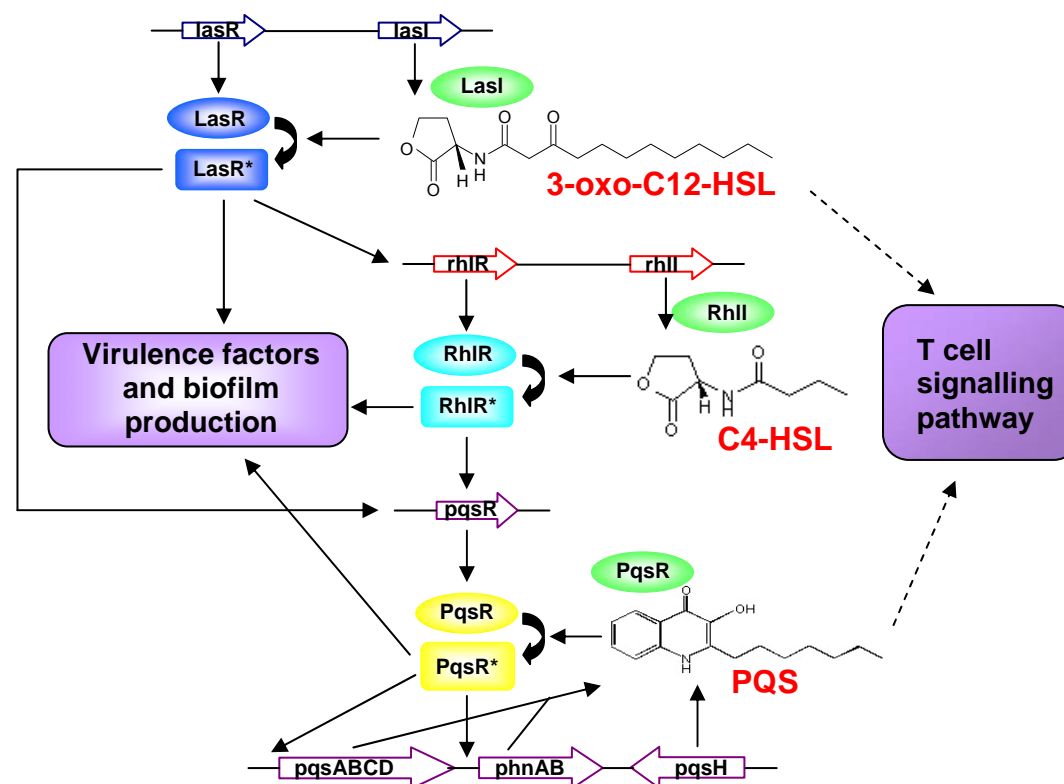
Figure 1.4. Simplified model of the *P. aeruginosa* quorum sensing hierarchy. As the bacterial population increases, AHLs accumulate, and once a maximum threshold is reached, these molecules bind to and activate their cognate transcriptional regulators, eventually leading to production of virulence factors and biofilm production. The AHL molecule 3-oxo-C12-HSL and the quinolones molecule PQS has been suggested to inhibit immune cell proliferation (Hooi *et al.*, 2004).

it appears to partition into cell membranes, and *P. aeruginosa* efflux pumps aid in the movement of this signal to the external environment (Pearson *et al*., 1999).

The second cell-to-cell signalling system, *rhl*, directs the synthesis of C4-HSL (fig. 1.4) (Pearson *et al.*, 1995, Winson *et al*., 1995). This diffusible signal (Pearson *et al.*, 1999) in conjunction with RhlR activates expression of the rhlAB rhamnolipid synthesis genes, rhamnolipid, and to some extent lasB, which encodes the major virulence factor LasB elastase (Gambello and Iglewski, 1991, Ochsner et al., 1994, Latifi et al., 1995, Ochsner and Reiser, 1995, Latifi et al., 1996, Pearson et al., 1997). Other virulence factors and secondary metabolites, including pyocyanin, cyanide, and chitinase are positively regulated by the *rhl* system (Latifi *et al*., 1995, Ochsner and Reiser, 1995).

Recent data have shown that the *las* and *rhl* QS systems of *P. aeruginosa* interact. Both systems are highly specific in that their respective autoinducers are unable to activate the transcriptional activator protein of the other system. However, neither system is completely independent of the other. The lasR/3-oxo-C12-HSL complex activates the expression of rhlR placing the *las* system in a cell-to-cell signalling hierarchy above the *rhl* system (Latifi *et al.*, 1996, Pesci and Iglewski, 1997). Moreover, 3-oxo-C12-HSL can bind to RhlR, blocking the binding of C4-HSL to its transcriptional activator rhlR (Pesci and Iglewski, 1997). The *las* system therefore controls the *rhl* system at both a transcriptional and post-translational level, and so genes controlled by the *rhl* system require a functional *las* system for full activation (fig. 1.4).

Studies have indicated that the production of PQS occurs only in the presence of an active form of lasR and the provision of exogenous PQS induces expression of lasB, *rhl* and rhlR which is important for PQS bioactivity (Pesci *et al.*, 1999, McKnight *et al.*, 2000). The discovery that PQS regulates the expression of lasB and that the synthesis and bioactivity of PQS are mediated by both the *las* and *rhl* QS systems, respectively, suggests that PQS and AHL-dependent QS must be interlinked (Pesci *et al.*, 1999, McKnight *et al.*, 2000). PQS strongly induces *rhl* in *P. aeruginosa* and has less positive effects on the transcription of lasR and rhlR. Furthermore, data indicates that *rhl* and lasB are both cooperatively regulated by PQS and C4-HSL, and the combined addition of C4-HSL and PQS has synergistic effect on the expression of lasB when compared to C4-HSL or PQS alone (McKnight *et al.*, 2000). However, McKnight *et al.* (2000) suggested that PQS is not involved in sensing cell density as it was identified much later in the growth cycle than is typical for QS signal molecule. It was speculated that the purpose of PQS in regulating *rhl* expression, might be to further up-regulate the *rhl* QS system in late stationary phase cultures i.e. at a time of increased cell stress (McKnight *et al.*, 2000). Conversely, studies have demonstrated that although PQS levels are indeed maximal in late stationary phase, they are already detectable in the logarithmic phase of growth, and that substantial levels of the molecule are present at the onset of stationary phase, indicating that PQS, apart from being important in late stationary phase may also have a function at a much earlier stage of growth (Diggle *et al.*, 2003, Lepine *et al.*, 2003).

More recently, the structural genes required for PQS have been identified (*pqsABCDH*) along with a transcriptional regulator (*pqsR*) and a response effector

(*pqsE*) (Gallagher *et al.*, 2002). The transcription of *pqsH* is regulated by the *las* QS system, linking QS and PQS regulation. Taken together, these data demonstrate that the PQS molecule is a central component of the *P. aeruginosa* QS hierarchy (fig. 1.4). Its addition was found to promote biofilm formation and to positively affect production of multiple quorum sensing-controlled virulence factors, including lectins, pyocyanin and proteases (Pesci *et al.*, 1999, McKnight *et al.*, 2000, Calfee *et al.*, 2001, Diggle *et al.*, 2003).

Work in the past few years showed that QS is essential for the expression of virulence factors as well as for biofilm formation by *P. aeruginosa* and thus represents an attractive target for the design of novel drugs for the treatment of *P. aeruginosa* infections.

1.5. Role of *P. aeruginosa* quorum sensing in pathogenesis

Taking into account that QS regulates such a wide range of factors that play such diverse roles in the function of *P. aeruginosa*, examining the role of QS in bacterial infections is inevitable. Studies confirm the importance of both the *las* and *rhl* QS systems for *P. aeruginosa* to disseminate from the initial site of infection which leads to septicaemia, induces both acute and chronic lung infections, and causes pathology and mortality (Rumbaugh *et al.*, 1999, Pearson *et al.*, 2000, Smith *et al.*, 2002). QS has also been shown to be functional during *P. aeruginosa* infections in humans. Sputum samples from CF patients chronically colonised with *P. aeruginosa* contained mRNA transcripts for the QS genes lasR and lasI (Storey *et al.*, 1998, Erickson *et al.*, 2002). Accumulation of these transcripts in sputum correlated with

the levels of transcripts for various QS-regulated genes. The first study to address the production of AHLs *in vivo* used mice infected with *P. aeruginosa* together with an *E. coli* AHL reporter strain. 3-oxo-C12-HSL produced by *P. aeruginosa* was detected in these mice directly demonstrating that *P. aeruginosa* AHLs were produced *in vivo* (Wu *et al.*, 2000). The production of AHLs *in vivo* was corroborated by studies that showed sputum from *P. aeruginosa* colonised CF patients was able to produce both 3-oxo-C12-HSL and C4-HSL (Singh *et al.*, 2000). In addition to these studies, AHLs were directly extracted and measured in the sputum of CF patients colonised with *P. aeruginosa* (Erickson *et al.*, 2002). Erickson *et al.* demonstrated the sputum of these CF patients contained approximately 22 nM 3-oxo-C12-HSL and 5 nM C4-HSL; however they speculated that these levels may be significantly lower than local concentrations associated with the bacteria (Erickson *et al.*, 2002). In experiments where *P. aeruginosa* were grown in a biofilm, concentrations of 3-oxo-C12-HSL were measured in association with the bacteria (Charlton *et al.*, 2000). These studies demonstrate that QS is active during *P. aeruginosa* infections, it potentially regulates the expression of various genes *in vivo* and that both 3-oxo-C12-HSL and C4-HSL are actively produced. The presence of the molecule *in vivo* may be a factor in allowing *P. aeruginosa* to develop or maintain a chronic state.

Furthermore, Collier *et al.* demonstrated that PQS was present in three different types of samples from infected CF patients. PQS production by *P. aeruginosa* cultured form chronically infected CF patients was observed where nine of the ten strains cultured form different patients produced varying amounts of PQS, leading to the conclusion that many *P. aeruginosa* CF strains are capable to producing PQS

when grown *in vitro* (Collier *et al.*, 2002). Coller *et al.* went onto analysing sputum from infected CF patients and showed the presence of PQS indicating that PQS is produced *in vivo*. Moreover, the amount of PQS evident in each extract correlates with the relative density of *P. aeruginosa* in each sample with the concentration of *P. aeruginosa* in the sputum sample containing the highest amount of PQS was approximately 2 μM. This concentration is on the edge of the physiologically active range but the authors speculated that this is an underestimate of the actual amount because of the inefficient PQS recovery achieved by organic extraction (Collier *et al.*, 2002, Pesci *et al.*, 1999). Since sputum must pass through the oral cavity and consists of a mixture of lower and upper respirator tract secretions, Collier *et al.* also examined samples collected below the larynx from patients infected with *P. aeruginosa*. Results indicated production of PQS in the bronchoalveolar space of CF patients as well as in mucopurulent airway fluid, providing additional evidence of PQS is produced inside the CF lung (Collier *et al.*, 2002)

The involvement of QS in *P. aeruginosa* biofilm formation has also been intensively studied where emphasis has been placed on PQS playing a major role in biofilm formation, protecting the bacterial cells from adverse environmental conditions including the host immune system and anti-microbial agents (Bjarnsholt and Givskov, 2006). It is generally appreciated that when *P. aeruginosa* chronically infects patients, it adapts to the biofilm mode of growth. The generally accepted definition of a biofilm is "a community of cells attached to a surface or interface or to each other, imbedded in a self-made, protective matrix of extracellular polymeric substances (EPS)". The clinical implications of bacterial biofilms are particularly pronounced (Davies, 2003). *P. aeruginosa* is capable of forming two general types

of biofilms in the laboratory based on their structure. A 'flat' biofilm is characterised by a relatively confluent, uniform community of bacteria on the surface. A 'structured' biofilm consists of cell aggregates or 'mushrooms' separated by channels or spaces (Kirisits and Parsek, 2006). The undifferentiated 'flat' biofilms are less stable than the differentiated biofilms as they can easily be disrupted by the detergent sodium dodecyl sulphate (Davies *et al.*, 1998). The ability of *P. aeruginosa* to form biofilms has severe implications for infected patients, as cells grown in biofilms are much more resistant against host defence systems and exhibit increased resistance against a variety of antibodies (Greenberg, 2003).

Davies *et al.* (1998) originally reported an involvement of the QS circuitry in the regulation of biofilm formation. In this study it was shown that the wild-type and rhlI mutant strains formed structured biofilms, while the lasI and the lasI/rhlI mutant strains formed flat, undifferentiated biofilms (Davies *et al.*, 1998). Based on this observation, it has been suggested that the *las* system is required for the development of typical biofilm architecture. Bjarnsholt *et al.* showed by viability staining and by determination of viable counts that a *P. aeruginosa in vitro* biofilm is much less tolerant of tobramycin treatment. The QS-deficient biofilm was almost entirely eradicated, in contrast with the wild-type biofilm, in which only cells in the top layer were killed (Bjarnsholt et al., 2005a). Shih and Huang obtained similar results by comparing wild-type and QS-deficient biofilms on treatment with kanamycin (Shih and Huang, 2002).

Favre-Bonté *et al.* determined that QSSMs are produced in biofilms covering intubation devices (Favre-Bonte *et al.*, 2007). The 3-oxo-C12-HSL and C4-HSL can

be detected *in situ* in biofilms covering intubation devices retrieved from patients colonised by *P. aeruginosa*. Moreover, all *P. aeruginosa* isolates collected either from these biofilms or from tracheal aspirates produced these QSSMs *in vitro*. Whereas 3-oxo-C12-HSL has been previously shown to play a role in the differentiation of *P. aeruginosa* biofilms (Davies *et al.*, 1998, De Kievit *et al.*, 2001), C4-HSL seems to be important during the maturation stage of biofilm development (Sauer *et al.*, 2002), for the total amount of biofilm formed (Favre-Bonte *et al.*, 2003), and for the maintenance of biofilm architecture (Davey *et al.*, 2003). In addition, Favre-Bonté *et al.* also demonstrated isolates recovered from the biofilm of intubation devices produced higher levels of C4-HSL than 3-oxo-C12-HSL *in vitro* (Favre-Bonte *et al.*, 2007) supporting previous observations where C4-HSL is produced in higher quantities than 3-oxo-C12-HSL in *P. aeruginosa* biofilms in the lungs of CF patients (Singh *et al.*, 2000, Favre-Bonte *et al.*, 2002).

Furthermore, PQS signalling has been shown to promote *P. aeruginosa* biofilm formation. Diggle *et al.* showed that *P. aeruginosa* biofilm development on stainless steel coupons was substantially enhanced in the presence of PQS. The stimulating influence of PQS on both RhlR/C4-HSL may be responsible for the biofilm phenotype observed in the presence of synthetic PQS (Diggle *et al.*, 2003).

A growing number of reports also indicate that autoinducers may efficiently enter and modulate gene expression in cells of a host eukaryotic organism (Williams *et al.*, 2004). Initial studies examining the effects of *P. aeruginosa* AHLs on eukaryotic cells used a strain containing a lasR deletion, which produces few AHLs. It was found that when this strain was added to *in vitro* culture of human bronchial

epithelial cells, the amount of IL-8 produced was significantly less than that stimulated by wild-type *P. aeruginosa* (Tang *et al.*, 1996). High levels of chemotactic factors, such as IL-8 and neutrophils, have been associated with chronic *P. aeruginosa* infections in CF patients. Neutrophils that enter the infection site secrete multiple mediators, such as reactive oxygen species and elastase. *P. aeruginosa* tends to be resistant to their effects likely to be due in part to the growth of the bacteria in protective biofilms. *P. aeruginosa* also produces catalases and superoxide dismutases that are able to neutralise the deleterious effects of hydrogen peroxide and oxidative stress (Hassett *et al.*, 1999). Therefore, instead of clearing the bacterial infection, the release of neutrophils mediators results in tissue destruction. Wagner *et al.* found that supernatants of *P. aeruginosa* collected within 2 to 24 hr after cultivating the bacteria under conditions leading to biofilm formation, contained a chemotactic activity for neutrophils (Wagner *et al.*, 2007). Chemotaxis and phagocytosis of bacteria is critically dependent on adhesion proteins. Wagner *et al.* tested the effect of 3-oxo-C12-HSL on the surface expression of the β2-integrin CD11b/CD18 on neutrophils and found that an up-regulation was seen, compatible with a transport to the membrane of performed molecules, as it occurs also in response to *bona fide* stimuli such as IL-8. Furthermore, an up-regulation of immunoglobulin (IgG) receptor CD16 was seen as was induction of CD64, the later representing the high-affinity receptor for IgG. Corresponding to the enhanced expression of the immunoglobulin receptors, an increased uptake of opsonised bacteria by 3-oxo-C12-HSL-pretreated neutrophils was seen (Wagner *et al.*, 2007).

It was also demonstrated that purified 3-oxo-C12-HSL at high concentrations (100 μM) activated p42/44 MAPK pathway and subsequently transcription factor

NFκB that stimulated the production of inflammatory cytokine IL-8 from human lung bronchial cells, but were unable to identify its binding target (Dimango *et al.*, 1995). Additional experiments examining this activation demonstrated a specific signal pathway that was stimulated by 3-oxo-C12-HSL in bronchial epithelial cells. 3-oxo-C12-HSL activated extracellular-signal-regulated kinases (ERKs) that subsequently induced the activation of the transcription factor NFκB. Thus activation of NFκB is essential for maximal production of IL-8 with 3-oxo-C12-HSL stimulation (Smith *et al.*, 2001). 3-oxo-C12-HSL was also found to induce the expression of several other chemokines that can stimulate the migration of monocytes, neutrophils and T cells (Smith *et al.*, 2002). These results were supported by *in vivo* experiments. Smith *et al.* showed that direct injection of 3-oxo-C12-HSL into the skin of C57BL/6 mice promoted the production of messenger RNA (mRNA) encoding cytokines IL-1α and IL-6 and macrophage inflammatory protein-2 (MIP-2), the mouse analogue of the human cytokine IL-8. This agrees with observation made by Bjarnoholt *et al.*, who found the MIP-2 concentration to be significantly higher in mice infected with QS active *P. aeruginosa* than those infected with the QS-deficient counterparts. They also observed that the high concentration of MIP-2 was correlated with slow clearance of the bacteria (Bjarnsholt *et al.*, 2005a, Bjarnsholt *et al.*, 2005b).

In addition, Vikström *et al.* investigated the influence of 3-oxo-C12-HSL on different functions of macrophages and specifically MAPK pathways in the cells (Vikstrom *et al.*, 2005). It was demonstrated that the level of phagocytic activity in macrophages was significantly higher after pre-treatment with 100 μM 3-oxo-C12-HSL for either 30 min or 1 hr with the authors speculating that the concentration and time exposure

constraints may help the macrophages to both sense and block *P. aeruginosa* biofilm formation by being stimulated to phagocytose the bacteria. Vikström *et al.* also provided evidence that 3-oxo-C12-HSL specifically activates the p38 MAPK pathway in human macrophages but failed to activate the p42/44 MAPK signalling cascade (Vikstrom *et al.*, 2005). Mitogenic stimuli, such as growth factors, activate the ERK or p42/44 cascades, whereas pro-inflammatory and stress stimuli activate the p38 and JNK pathway. SB203580 is a specific inhibitor of p38 MAPK, and has thus been widely used to investigate the role of p38 MAPK in the function and differentiation of cells (Kurosaka *et al.*, 2003, Pearson *et al.*, 2001, Rao, 2001, Carter *et al.*, 1999, Dziarski *et al.*, 1996). Vikström *et al.* also found that pre-treatment of cells with 10 μM SB203580 prevented 3-oxo-C12-HSL-induced phagocytic activation of human macrophages. These findings demonstrate the notion that p38 MAPK cascade is involved in 3-oxo-C12-HSL-induced increase of phagocytic capacity (Vikstrom *et al.*, 2005). In contrast to these observations, it was previously shown that 3-oxo-C12-HSL can induce rapid activation of p42/p44 and subsequently the NFκB pathway in human lung fibroblasts and epithelial cells but failing to activate the p38 MAPK pathway (Smith *et al.*, 2001), speculating that 3-oxo-C12-HSL can distinctly modulate MAPK signalling cascades in different cell types.

In contrast to the findings that 10 μM and 100 μM of 3-oxo-C12-HSL did not induce cell death of neutrophils from human peripheral blood (Wagner *et al.*, 2007), and enhancement of macrophage activity with 100 μM 3-oxo-C12-HSL (Vikstrom *et al.*, 2005), Tadeta *et al.* reported induction of apoptosis in mouse macrophages and in neutrophils with 50 μM of 3-oxo-C12-HSL (Tateda *et al.*, 2003). Species and

concentration differences apparently account for the discrepant finding, as apoptosis was not observed in human cells.

Smith *et al* also reported that 3-oxo-C12-HSL can up-regulate the expression of cyclooxygenase-2 (cox-2), an enzyme important for the production of prostaglandins (Smith *et al.*, 2002).   This induction of cox-2 and subsequent activation of prostaglandin E2 (PGE$_2$) synthase resulted in the increased production of PGE$_2$. Increased levels of PGE$_2$ have been associated with *P. aeruginosa* infections in CF lungs an in burn wounds (Hahn *et al.*, 1999, Konstan *et al.*, 1993).   PGE$_2$ has many functions, one of which is the induction of endothelial permeability.   Compounding this response is the inhibition of both coronary and pulmonary smooth muscle contractions with 3-oxo-C12-HSL stimulation (Lawrence *et al.*, 1999).   This vasorelaxant activity might lead to increased local blood flow, oedema and cellular migration to the site of infection.

Studies also demonstrated that relatively high concentrations of 3-oxo-C12-HSL (in the 1-100 μM range) (but not the short chain C6-HSL) could inhibit the production of IL-12 and TNFα from LPS-activated mouse peritoneal exudates cells or hPBMCs (Telford *et al*., 1998, Chhabra *et al.*, 2003).   IL-12 is instrumental in the stimulation of IFNγ production from T cells, which is important for the activation of macrophages and the induction of proinflammatory response.   Down-regulation of IL-12 may support the Th2-dominated adaptive immune response which is characteristic of CF patients with chronic *P. aeruginosa* lung infection (Moser *et al.*, 2000).   This inhibition of IL-12 could potentially alter the activation of T cells and the production of IFNγ (Telford *et al.*, 1998).   In contrast, Smith *et al.* demonstrated

3-oxo-C12-HSL can directly interact with T cells and stimulate the production of IFNγ with little or no effect on IL-4 in transgenic murine splenic T cells (Smith *et al.*, 2002), though both studies are not totally contradictory as the concentration of 3-oxo-C12-HSL used by Telford *et al.* were high (~ 100 μM) compared to Smith *et al.* (< 10 μM).

In 2003, Richie *et al.* showed that 3-oxo-C12-HSL alone promotes neither Th1 nor Th2 response when administered to mice (Ritchie *et al.*, 2003). The authors hypothesised that 3-oxo-C12-HSL interacts non-specifically with the host innate immune response, and this could be by direct interaction with the leukocytes and not by interfering directly with the cytokine profile and other processes in the inflammation cascade. It was concluded that caution is required when extrapolating to *in vivo* scenarios the effects of 3-oxo-C12-HSL obtained *in vitro*, because the response is greatly affected by the immune status of the host.

Additionally, Hooi *et al.* demonstrated that 3-oxo-C12-HSL and PQS can exert differential modulatory effects on mammalian immune responses *in vitro* (Hooi *et al.*, 2004). PQS and 3-oxo-C12-HSL significantly reduced the ability of human peripheral blood mononuclear cells (hPBMCs) to respond to ConA and to anti-CD3 and anti-CD28. PQS did so without any effect on cell viability while 3-oxo-C12-HSL suppressed proliferation before viability was affected. Furthermore, 3-oxo-C12-HSL was shown to inhibit IL-2 and TNFα release while PQS stimulated the release of these cytokines (Hooi *et al.*, 2004). This paper described for the first time the immune-modulatory activity of PQS and demonstrated the different immunological activities of these QSSMs.

Given the documented immune modulatory activity of 3-oxo-C12-HSL, especially its ability to inhibit TNFα and IL-12 and so promoting Th2 responses (Telford *et al.*, 1998), Pritchard *et al.* investigated its effect on the development of insulitis and diabetes in non-obese diabetic (NOD) mouse (Pritchard *et al.*, 2005). NOD mouse is a well established model of type 1 insulin-dependent diabetes mellitus and studies have demonstrated the immune response leading to β-cell destruction is characterised by a Th1 type CD4 T cell response and involves TNFα (Christen *et al.*, 2001). β-cells in the islets of Langerhans of the pancreas are required for insulin production. Blockage of IL-12 production has also been shown to inhibit the development of diabetes (Falcone *et al.*, 1999). Pritchard *et al.* demonstrated that 3-oxo-C12-HSL significantly reduced insulitis and NOD mice treated with 3-oxo-C12-HSL was shown to significantly retard the cumulative incidence of diabetes, suggesting 3-oxo-C12-HSL treated mice would be protected from the development of diabetes (Pritchard *et al.*, 2005). This study indicates the potential for QSSMs to be used therapeutically.

Additional studies characterising this response in leukocytes observed that the structure of the AHL molecule is important for the regulation of cytokine production. Work by Chhabra *et al.* (2003) showed that inhibition of ConA-stimulated murine spleen cell proliferation by a range of 3-oxo-C12-HSL-related compounds required an acyl side chain a minimum of 11 carbons in length and high lipophilicity of the molecule. The homoserine ring was also shown to be essential for efficient suppression of lymphocyte proliferation (fig. 1.5a) (Chhabra *et al.*, 2003). Dimango *et al.* determined C6-HSL and C4-HSL were unable to stimulate production of IL-8 in human lung bronchial epithelial cell, indicating the structure of 3-oxo-C12-HSL

was important for this activation (Dimango *et al.*, 1995). In addition to AHLs, the activity of PQS also depends on the presence of the 3-hydroxy group as neither 2-heptyl-4(1H)-quinolone nor 3-formyl-2-heptyl-4(1H)-quinolone exhibited any biological activity comparable with PQS (fig. 1.5b) (Diggle *et al.*, 2003). Thus, it seems likely that the PQS signal transduction pathway contains a sensor protein that recognises PQS with a high degree of structural specificity.
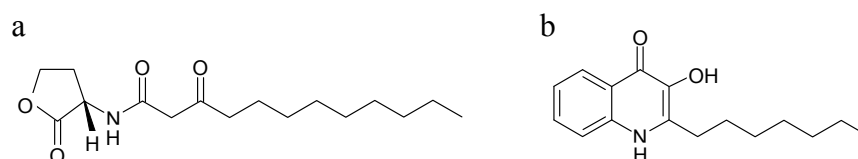
Figure 1.5. Structure of 3-oxo-C12-HSL (a) and PQS (b)

In addition to gram-negative bacteria, a number of gram-positive bacteria are known to employ quorum-sensing systems. No gram-positive bacteria have been shown to produce AHLs; gram-positive quorum sensing systems typically make use of small posttranslationally modified peptide-based signal molecules. Quorum sensing is used to regulate the development of bacterial competence in *Bacilllus subtilis* and *Streptococcus pneumoniae*, conjugation in *Enterococcus faecalis* and virulence in *Staphylococcus aureus* and *Enterococcus faecalis* (Dunny and Leonard, 1997, Kleerebezem et al., 1997, Novick and Muir, 1999).

The virulence of *S. aureus* is dependent on the temporal expression of a diverse array of virulence factors regulated by the *agr* locus (Novick and Muir, 1999). During the early stages of *S. aureus* infection, surface proteins involved in attachment and defence e.g. protein A, coagulase and fibronectin-binding proteins predominate. However, once a high cell density is achieved at the infection site, expression of

S. aureus surface proteins is decreased and secreted protein are preferentially expressed e.g. proteases, hemolysins, toxic shock syndrome toxin-1 and enterotoxin B (Dunny and Leonard, 1997, Bronner *et al.*, 2004, Novick and Muir, 1999). In several experimental animal models of *S. aureus* infection, *agr* mutants exhibit significantly reduced virulence, highlighting the key role of this regulatory locus in staphylococcal pathogenicity (Cheung *et al.*, 2004).

The growing number of human pathogens found to contain QS systems highlights the importance of exploring further into this area and increasing our understanding of the mechanisms involved, allowing future exploitation of these regulatory systems for therapeutic interventions.

1.6. Microarray technology

As the human genome is gradually fully sequenced, and protein interaction maps become available, the scientific community is able to evaluate the complexity of the eukaryotic cellular network. To take advantage of this knowledge, further studying the differential effects of 3-oxo-C12-HSL and PQS at the mRNA level would be valuable in assessing molecular targets within the immune system. The examination of gene expression using microarrays holds tremendous promise for the identification of candidate genes involved in a variety of processes. Gene expression is a central concept in molecular biology and forms part of our knowledge of the role of genes in human diseases. Genome-wide monitoring of gene expression using DNA microarrays represents one of the latest break-through in experimental molecular biology and provides unprecedented opportunity to explore changes in cell signalling

pathways when faced with bacterial infections by providing a comprehensive survey of a cell's transcriptional landscape. Gene microarray technology has become highly valuable for identifying complex global changes in gene expression patterns, by harvesting total cellular RNA and using it to generate probes through a variety of strategies including incorporating a label into the first strand cDNA made from total RNA or attaching a T7 RNA polymerase promoter during cDNA synthesis, then labelling of the resulting RNA (fig. 1.6).

Microarray therefore allows simultaneous assessment of the transcription of tens of thousands of genes, and of their relative expression between 'treated' cells and 'untreated' cells. Cristillo and Bierer used cDNA-based microarray analysis to obtain a more comprehensive view of CsA- and FK506-sensitive early genes in purified human peripheral blood T lymphocytes (Cristillo and Bierer, 2002). Cristillo and Bierer hypothesised that changes in gene expression common to both CsA and FK506 treatment would likely be secondary to inactivation of calcineurin phosphatase activity. Additionally, perturbations in gene expression unique to one drug alone would potentially identify specific immunophilin (CyP or FKBP)-dependent gene targets. Although Cristillo and Bierer failed to identify transcripts specifically regulated by only one immunosuppressive agent, suggesting that immunophilin-dependent, calcineurin-independent gene expression was below the limits of detection of their analysis, they did identify a number of CsA- and FK506-sensitive (and calcineurin-dependent) genes induced or inhibited following anti-CD3 monoclonal antibody ligation relative to resting cells (Cristillo and Bierer, 2002).

Figure 1.6
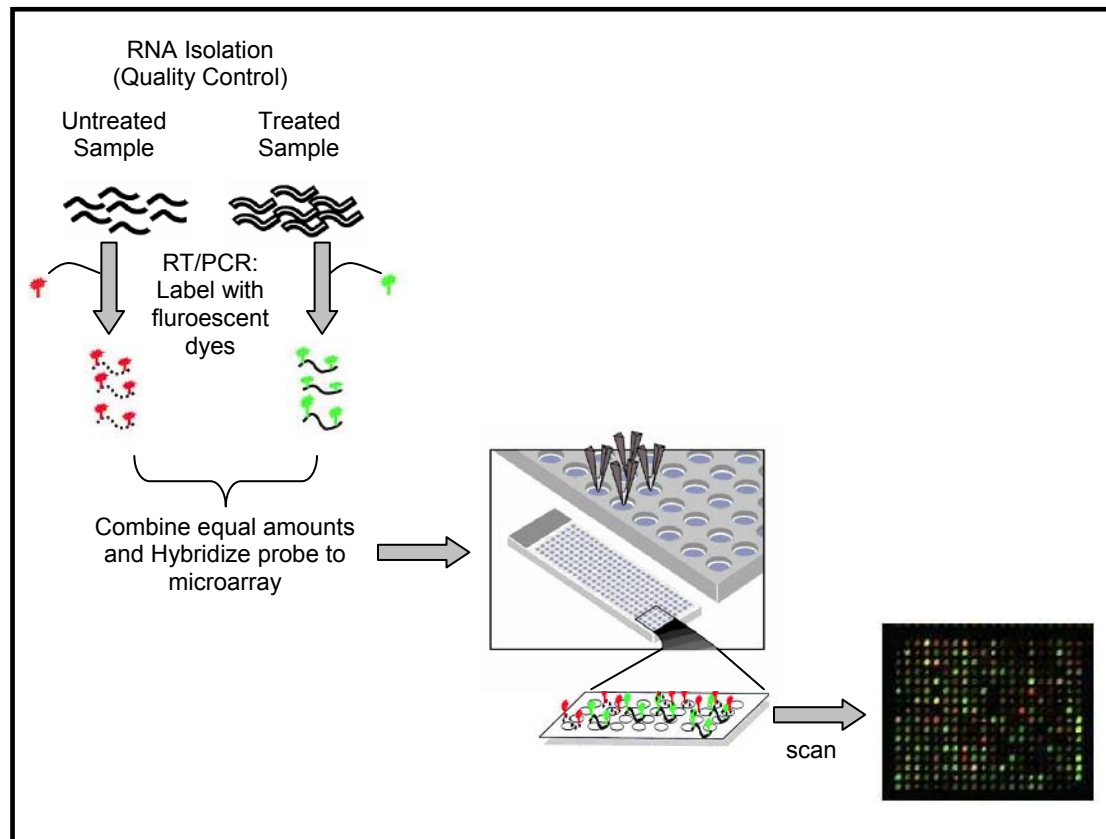
Figure 1.6. Schematic outline of a microarray analysis experiment. RNA is isolated from reference (control) cells and test (experimental) cells. cDNA is synthesised from each RNA population by a reverse transcription process. The resulting two cDNA samples (control and experimental) are labelled with two different fluorescent dyes, mixed, and hybridized to the targets the microarray. Hybridization is monitored through measurement of the amount of fluorescence associated with individual target molecules on the array. Since the two dyes fluoresce at different wavelengths, it is possible to distinguish hybridization due to the control cDNA population from that due to the experimental cDNA population (adapted from http://www.microarray.lu/en/MICROARRAY_Overview.shtml, http://www.genome.gov/10000533).

Microarray analysis was also used by Grolleau *et al.* to identify global and specific effects of rapamycin on translation. Translation of a large number of ribosomal proteins and elongation factors were found to be strongly repressed by rapamycin and a large number of additional genes uncovered. Part of the regulated genes has functions related to RNA processing and translation, for example, translation initiation factors 4A and 5A were strongly repressed and translation of prothymosin alpha was also strongly repressed by rapamycin (Grolleau *et al.*, 2002). Prothymosin alpha has been reported to enhance cell-mediated immunity as well as proliferative and cytotoxic responses of T cells (Cordero *et al.*, 1992, Bustelo *et al.*, 1991).

Comparing differences in total RNA expression levels between treated T cells, subjected to known immunosuppressive agents or QSSMs, and control T cells stimulated to proliferate using anti-CD3 and anti-CD28 on a 30K human array, may therefore give greater insights to the effects QSSMs have at a genetic level and comparing these effects to currently used immunosuppressive drugs.

1.7. Summary

In summary, quorum sensing is important for the regulation of multiple factors in *P. aeruginosa* that has a significant effect on the "virulence" of the bacteria. Moreover, the production of 3-oxo-C12-HSL and PQS by *P. aeruginosa* is not only important for cell-to-cell communication between the bacteria, but also directly acts as a virulence factor. It is clear from previous studies that 3-oxo-C12-HSL can inhibit (at low doses) and activate as well as cause apoptosis (at high doses) in multiple cell types and thus potentially change the host response during *P. aeruginosa* infections (Telford *et al.*, 1998, Smith and Iglewski, 2003a, Smith and Iglewski, 2003b). It has also been recently demonstrated that PQS is also immunosuppressive in hPBMCs, and these two QSSMs engage different molecular targets within the cells, establishing differential effects – 3-oxo-C12-HSL showing an inhibitory effect while PQS showing a stimulatory effect on IL-2 and TNFα production (Hooi *et al.*, 2004, Pritchard, 2006).

The clinical implications of these findings are potentially significant, as the differential activity reported for 3-oxo-C12-HSL and PQS suggest that QSSMs act immunologically in infected tissues and supports the concept that QSSMs are true virulence determinants (Collier *et al.*, 2002). Increasing the understanding of immune modulation by these two QSSMs is important. Present studies have focused on their effects on murine splenocytes and hPBMCs.

The purpose of my research was to study for the first time the effects of QSSMs on pure human T cells, shown to be important in immunity to *P. aeruginosa* (Stevenson et al., 1995). This study confirms the differential effects of 3-oxo-C12-HSL and

PQS on IL-2 and TNFα release from pure T cells isolated from whole blood. In addition, to further understand the mechanism of actions of these two QSSMs, the effect they have on IFNγ and IL-4 was also tested, as they are markers of Th1 and Th2 responses respectively. Further cytokine analysis was performed to confirm the effect the two QSSMs have on Th1 and Th2 responses. The differential effects of 3-oxo-C12-HSL and PQS observed in pure human T cells supports previous suggestions they may act in a similar manner to CsA and rapamycin. Establishing the effects these two QSSMs have on pure T cells help to further understand their mechanisms of actions and possibly contributing to future development of novel therapeutics for a multitude of immunological diseases.

1.8. Aims and Objectives

To further understand the interactions of QSSMs within the immune system by assessing:

- The differential effects of 3-oxo-C12-HSL and PQS on mouse splenocyte proliferation and IL-4 and IFNγ release.

- The differential effects of 3-oxo-C12-HSL and PQS on human PBMC cell proliferation and IL-2 release.

- The differential effects of 3-oxo-C12-HSL and PQS on pure human T cell proliferation and IL-2 release.

- The effects *P. aeruginosa* QSSMs on an array of cytokine expression in pure human T cells using cytometric bead analysis technology.

- The effect of PQS on total and phospho-Erk 1/2 activity in pure human T cells.

Proceed to investigate at a gene array level the differential effects of *Pseudomonas* derived and classical immune suppressive chemicals on total RNA expression following engagement of the TCR:

- Isolate total RNA from pure T cells stimulated to proliferate using anti-CD3 and anti-CD28 and treated with either 3-oxo-C12-HSL, PQS or CsA using the IC_{50} concentration values obtained from the proliferation assays.

- Assessment of total RNA quality and quantity using Agilent 2100 Bioanalyzer in combination with Nanodrop spectrophotometer.

- RNA amplification and dye incorporation using Ambion's MessageAmp^{TM} aRNA Amplification kit followed by quality control.

- Microarray hybridisation onto a MWG 30K human array, scanning is performed using an Axon GenePix 4000B scanner and the scanning output file imported into Genespring (Silicon genetics) to quality control and analyse data.

2. General Materials and Methods

2.1. Preparation of QSSMs and CsA

The 3-oxo-C12-HSL and PQS used for all the procedures were synthesised by the Medicinal Chemistry group, University of Nottingham, according to the method described by Chhabra *et al.* (1993). The compounds were purified to homogenecity by semi-preparative high performance liquid chromatography and their structures confirmed by mass spectrometry and proton nuclear magnetic resonance spectroscopy (Chhabra *et al.*, 1993, Winson *et al.*, 1995). 3-oxo-C12-HSL and PQS were dissolved in 100% dimethyl sulphoxide (DMSO) at a concentration of 0.1 M and stored at 4°C.

CsA (Sigma, Poole, UK) was solubilised in 100% ethanol at a concentration of 0.05 M and stored at -20°C in 10 µl aliquots.

2.2. Cell culture

Mouse splenocytes, human PBMC and human T-lymphocytes were maintained in complete cell culture medium (CTCM) consisting of endotoxins-free RPMI 1640 supplemented with 5% heat inactivated fetal calf serum (FCS, Sigma, Poole, UK), 100 U/ml penicillin/100 µg/ml streptomycin (Sigma, Poole, UK), 2 mM L-glutamine (Sigma, Poole, UK) and $5x10^{-5}$ M 2-meracaptoethanol (ME, Sigma, Poole, UK).

2.3. Cell density determination

Cell density and viability were determined by trypan blue (Sigma, Poole, UK) dye exclusion. This method is based on the principle that viable cells are not permeable to certain dyes, whereas non-viable cells are. Cell suspension was mixed thoroughly via pipetting before adding 5 µl of cell suspension to 20 µl of trypan blue solution (0.4% (*w/v*) trypan blue in PBS). The trypan blue-cell suspension was transferred to both chambers of the haemocytometer without overfilling the chambers. Viable cells were counted within the large middle square. Cell concentration per ml and the total number of cells were determined using the following calculations:

Cells per ml   = Total cell count x 10^4 x 5 (dilution factor)

Total cell no.   = Cells per ml x original vol. of cell suspension

2.4. Cytotoxicity assay

Cell viability was determined using a colorimetric method, utilising solutions of a novel tetrazolium compound 3-(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonphenyl)-2H-tetrazolium, inner salt (MTS, Promega, Southampton, UK) and an electron-coupling reagent phenozine methosulphate (PMS, Promega, Southampton, UK). The MTS-PMS mix is bio-reduced by viable cells into a formazan product that is soluble in tissue culture medium (Barltrop *et al.*, 1991, Cory *et al.*, 1991, Riss and Moravec, 1992). The MTS-PMS mix is versatile and offers several advantages over MTT tetrazolium and other cytotoxicity assays due to the solubility of the MTS-PMS formazan product in

tissue culture medium and its specificity for living cells. The production of the coloured formazan product is thus proportional to the number of viable cells in culture.

Briefly, 100 µl cells suspension was added at a density of 10^5 cells per well of 96-well flat-bottomed tissue culture plates (NUNC, Leicestershire, UK) with either 3-oxo-C12-HSL, PQS or CsA at concentrations of 100 µM and subsequent half dilutions, and incubated for 48 hr at 37°C in a humidified, 5% CO_2 atmosphere. 40 µl MTS/PMS solution was then added per 200 µl cell culture for 4 hr at 37°C in 5% CO_2-air, protected from light. The production of formazan was determined by measuring the absorbance of the compound at 490 nm with a spectrophotometric 96-well plate reader (Dynex Technologies, Sussex, UK). The viability of the cells was calculated as follows:

% viability = ([absorbance of treated cells]/[absorbance of untreated cells]) x 100

2.5. Cytokine determination using ELSIA

The levels of interferon-γ (IFNγ) and interleukin-4 (IL-4) (BD Pharmingen, UK) produced in the murine splenocyte culture supernatants, and the levels of IL-2 (BD Pharmingen, UK) produced in the hPBMC and pure T cell culture supernatants after 24 hr were determined using 'sandwich' Enzyme-Linked Immunosorbent Assay (ELISA). Briefly, 96-well Nunc MaxiSorp (Life Technologies, Paisley, UK) plates were coated overnight at 4°C with 100 µl of purified anti-cytokine capture antibody per well (BD Pharmingen, UK) at 2 µg/ml diluted with binding solution (0.1 M

Na_2HPO_4, adjusted to pH 9 with 0.1 M NaH_2PO_4). After washing the plates three times with PBS-Tween (Phosphate buffered saline, with 0.5% (*v/v*) Tween-20 (Sigma, Poole, UK)), plates were blocked at room temperature (RT) for 2 hr with 200 µl/well concentration of 1% (*w/v*) BSA (Sigma, Poole, UK) in PBS. Following four washes with PBS-Tween, 50 µl of cell culture supernatants were added and incubated overnight (o/n) at 4°C; recombinant cytokines (BD Pharmingen, UK) were included as standards for each plate. After washing the plates four times with PBS-Tween, 100 µl/well of biotinylated detection antibody (1:250 dilution) (BD Pharmingen, UK) were added at 1 µg/ml for 1 hr at room temperature then washed for a further 4 times. The presence of biotinylated antibodies was detected by the addition of 100 µl/well of a 1:1000 dilution streptavidin-peroxidase (BD Pharmingen, UK) and 30 min incubation at RT. After thorough washes the assay was developed using 100 µl of 0.1 mg/ml 3,3',5,5'-tetramethylbenzidine substrate (Sigma, Poole, UK) in 0.05 M phosphate-citrate buffer (pH 5), containing 0.03% hydrogen peroxide. The enzyme reaction was stopped with 50 µl of 2 M sulphuric acid (Fisher Scientific Ltd, UK) after an incubation of 15 min at RT, and the colorimetric development was read at 450 nm with a spectrophotometric 96-well plate reader (Dynex Technologies, Sussex, UK). Cytokine levels were determined by interpolation from the reference standard curve obtained from recombinant cytokines.

2.6. Statistical analysis

Statistical analysis all data were performed by applying the parametric, ordinary analysis of variance (ANOVA) test, followed by Dunnetts' test, comparing each of

the treatment groups with control groups. Probability values (*P*-value) of <0.001, <0.01 or <0.05 were considered significant with 99.9 %, 99 % or 95 % of confidence respectively.   All calculations were performed with Sigmastat version 3.5.

Values for 50% inhibitory concentration (IC_{50}) were calculated from nonlinear regression analysis by using Graph-Pad Prism version 5 for Windows.

3. Assessment of the effect of 3-oxo-C12-HSL, PQS and CsA on mouse splenocyte proliferation and cytokine production

3.1. Introduction

The *P. aeruginosa* signalling molecules 3-oxo-C12-HSL and PQS are known to suppress mouse splenocyte proliferation (Chhabra *et al.*, 2003). In addition, 3-oxo-C12-HSL is known to affect cell viability at high concentrations (Telford *et al.*, 1998, Chhabra *et al.*, 2003, Hooi *et al.*, 2004). The original report of immunomodulation by Telford *et al.* demonstrated an inhibition in production of the Th1 cytokines TNFα and IL-12 by murine peritoneal macrophages from BALB/c mice stimulated with LPS *in vitro* by 3-oxo-C12-HSL at concentrations > 70 μM (Telford *et al.*, 1998). In contrast, Smith *et al.* saw a significant induction in the production of IFNγ by 5 μM 3-oxo-C12-HSL, at levels similar to those induced by the cytokine IL-12, but had no effect on IL-4 production, suggesting 3-oxo-C12-HSL induces an inflammatory Th1 phenotype in T cells (Smith *et al.*, 2002). Moreover, Ritchie *et al.* demonstrated that *in vitro* exposure to 3-oxo-C12-HSL had a suppressive effect on both IFNγ and IL-4 protein secretion by mitogen and antigen stimulation of mouse splenocytes at concentrations ranging from 1-10 μM (Ritchie *et al.*, 2003).

The aim of this chapter was to confirm the effects of 3-oxo-C12-HSL and PQS on mouse splenocytes stimulated with ConA or anti-CD3. Traditionally, mitogenic lectins such as ConA have been used for polyclonal T cell stimulation. ConA activate T cells by binding to cell membrane glycoproteins, including the TCR-CD3

complex (Kay, 1991). ConA is reported to stimulate cytotoxic T cells (Simon *et al.*, 1986), suppressor inducer T cells (Morimoto *et al.*, 1985), or 'virgin' T cells (Miller *et al.*, 1991). The effects of ConA on activation and proliferation of mature T cells are well known and similar to those induced by anti-CD3 antibody, mimicking the effects of high avidity of TCR ligation. (Powell and Leon, 1970, Novogrod.A and Katchals.E, 1971). However, a more physiologically relevant approach uses anti-CD3 and anti-CD28 to stimulate T cells in a manner that partially mimics stimulation by antigen-presenting cells (Trickett and Kwan, 2003). Anti-CD3 provides an initial activation signal to T cells, but proliferation is dependent on co-stimulatory signal, usually via the CD28 molecule (Acuto and Michel, 2003). Interestingly, anti-CD28 is not required to stimulate murine lymphocyte proliferation. Studies have demonstrated T cells from CD28-deficient mice are still able to produce immune responses to pathogens (Shahinian *et al.*, 1993, Green *et al.*, 1994) thus anti-CD28 was not used to stimulate murine proliferation in this study.

3.2. Methods

To understand the effects of 3-oxo-C12-HSL and PQS on murine splenocytes, cell proliferation, viability and cytokine production were examined.

3.2.1. Mouse splenocyte isolation and stimulation

Eight-week-old female BALB/c mice were obtained from Harlan (Bicester, Oxon, UK) and given food and water *ad libitum*. The ConA (Sigma, Poole, UK) or anti-CD3 (BD Pharmingen) proliferation assay was used to assess the effect of *P. aeruginosa* QSSMs (3-oxo-C12-HSL and PQS, provided by Prof. David Pritchard, University of Nottingham, UK) and CsA (Sigma, Poole, UK) on splenocyte activation and proliferation. The concentration of ConA (Sigma, Poole, UK) and anti-CD3 (BD Pharmingen, UK) that induced optimal spleen cell proliferation was determined in preliminary dose response experiments with concentrations ranging from 0.03 μg/ml to 4 μg/ml and 0.04 μg/ml to 6 μg/ml respectively. Proliferation was assessed by the incorporation of [³H]-thymidine (Amersham Biosciences) into DNA.

Splenocyte suspensions were prepared by removing the spleens aseptically and placing them in cold RPMI 1640 (Roswell Park Memorial Institute, Sigma, Poole, UK). Briefly, spleens were disrupted by forcing through a 70 μM nylon cell strainer (BD Biosciences, Europe) with a plunger from a 5 ml syringe to produce a single cell suspension in complete cell culture medium (CTCM). Cells were pelleted by centrifugation at 600 x *g* for 10 min, re-suspended, and erythrocytes lysed by

treatment with lysis buffer (0.017 M Tris (Sigma, Poole, UK), 0.144 M ammonium chloride buffer (Sigma, Poole, UK), adjusted to pH 7.2) for 5 min at room temperature (RT). Cells were washed in RPMI 1640 medium with 2% (*v/v*) FCS, pelleted by centrifugation and subjected to a second treatment with lysis buffer and washed twice before re-suspending in 10 ml CTCM. Cell density and viability were determined using the trypan blue assay. 100 µl cell suspension (10^5 cells) was added per well of 96-well round-bottom tissue culture plates.

3-oxo-C12-HSL, PQS and CsA were tested at final concentrations of 100 µM and subsequent half dilutions with either 1 µg/ml ConA or 3 µg/ml anti-CD3. Experiments were performed in triplicate wells in a final volume of 200 µl per well. After 24 hr incubation at 37^oC under 5% CO_2, 100 µl of cell culture supernatants was removed and stored at -20^oC until required for cytokine analysis.

3.2.2. Proliferation assay

[^{3}H]-thymidine incorporation into cells is a convenient method for quantifying cell proliferation, reflecting DNA synthesis. DNA synthesis is measured by incorporation of the radioactive exogenous precursor [^{3}H]-thymidine into proliferating cells. Briefly, following a further 24 hr incubation cultures were pulsed for 24 hr with 10 µl of 0.25 µCi/well ^{3}H-thymidine (Amersham Pharmacia Biotech. Inc., UK). Cells were then harvested onto 96-well glass fibre culture (G/F C) plates (Unifilter Filtermate Harvester^{TM}, Packard Bioscience Ltd, UK) left to dry at 37^oC, followed by the addition of 40 µl scintillant (MicroScint-O^{TM}, Packard Bioscience Ltd, UK) to each well. The radioactivity in the filters was measured for 2 min per

filter with a β-scintillation counter (Topcount[TM], Packard Bioscience Ltd, UK) according to manufacturers' specification. The results are expressed as the mean cpm of [^{3}H]-thymidine incorporated in triplicate cultures ± SD (Appendix 2) and mean cpm of all individual data ± SE (main chapter).

3.3. Results

3.3.1. Optimisation of assay condition

The dose response of ConA and anti-CD3 were initially assessed stimulating mouse splenocytes with 4 μM ConA and subsequent half dilutions or 6 μM anti-CD3 and subsequent half dilutions. Both ConA and anti-CD3 stimulated cellular proliferation in a dose dependent manner. The optimal dose for ConA was 1 μg/ml (fig. 3.1a), while the optimal dose for anti-CD3 was 3 μg/ml (fig. 3.1b).

3.3.2. Effects of QSSMs and CsA on ConA stimulated cell proliferation

Both PQS and 3-oxo-C12-HSL significantly inhibited cell proliferation in a dose dependent manner when splenocytes isolated from BALB/c mice spleens were stimulated with 1 μg/ml ConA (fig. 3.2a). PQS exhibited an IC_{50} value of 2.3±0.06 μM compared to 6.3±0.4 μM for 3-oxo-C12-HSL. CsA also inhibited splenocyte proliferation giving an IC_{50} value of 2.8±0.7 μM. DMSO and ethanol were used as diluents controls and did not exert any significant effect on the ConA stimulated cell proliferation.

3.3.3. Effects of QSSMs and CsA on anti-CD3 stimulated cell proliferation

As with mouse splenocytes stimulated with ConA, both PQS and 3-oxo-C12-HSL inhibited cell proliferation in a dose dependent manner when spleen cells were stimulated with 3 μM anti-CD3 (fig. 3.2b). Similar to the ConA proliferation assays,

PQS exhibited an IC_{50} value of 2.3±0.1 μM compared to that of 5.7±0.4 μM for 3-oxo-C12-HSL. CsA also significantly suppressed anti-CD3 driven splenocyte proliferation with an IC_{50} value of 2.1±0.3 μM, similar to the IC_{50} value of PQS. DMSO and ethanol did not exert any significant effect on the cell proliferation.

Figure 3.1

a

b
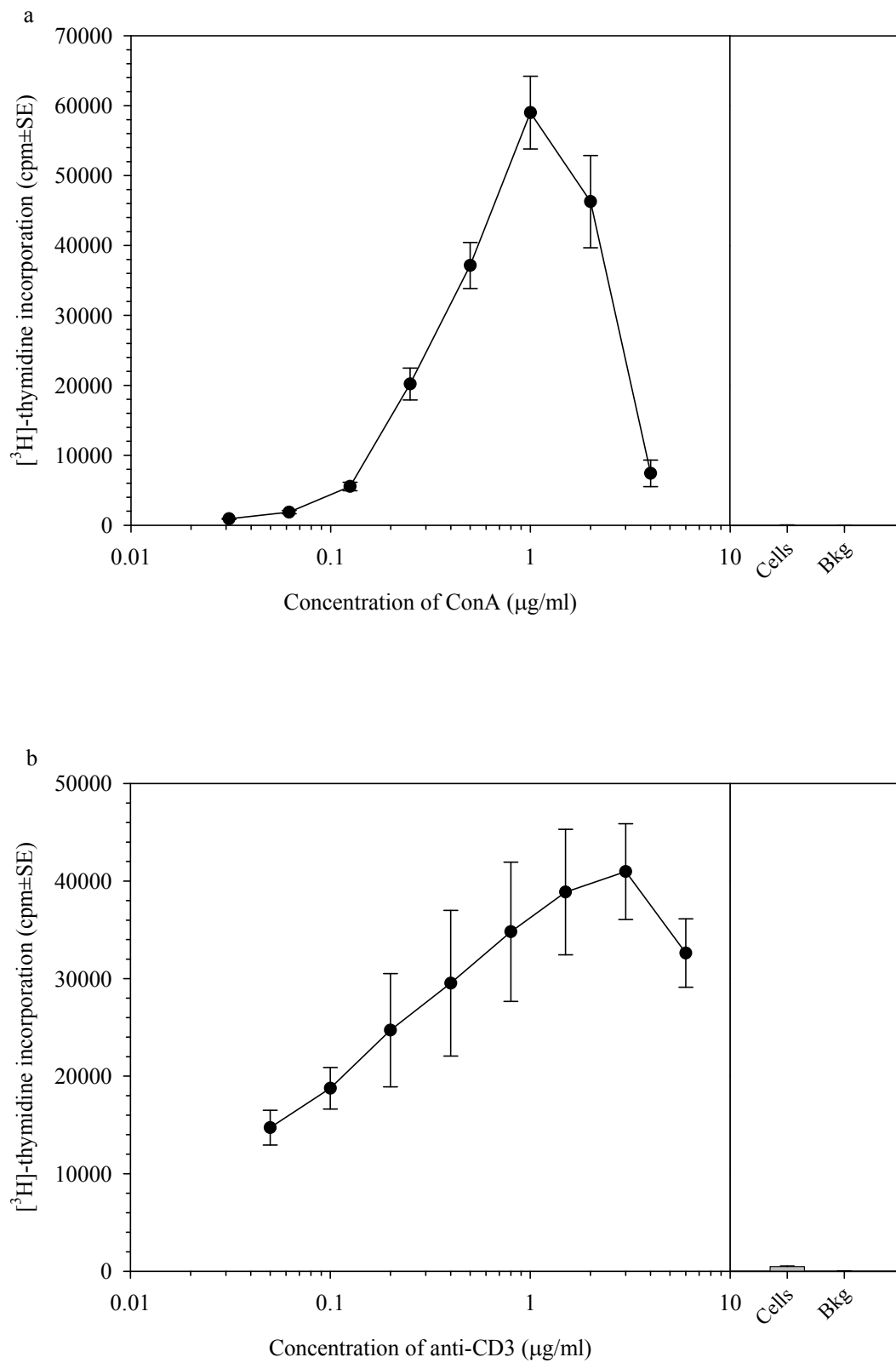
Figure 3.1. **Stimulatory effect of ConA (a) and anti-CD3 (b) on murine splenocyte.** ConA and anti-CD3 stimulated cellular proliferation in a dose dependent manner compared to the cell control (untreated cells) with an optimal dose of 1 μg/ml and 3 μg/ml respectively. Bkg (background) represents readings for CTCM. Data represents the mean of 6 and 4 independent experiments respectively; the results of the original experiments are individually plotted in A.2.1 for ConA and A.2.2 for anti-CD3.

Figure 3.2
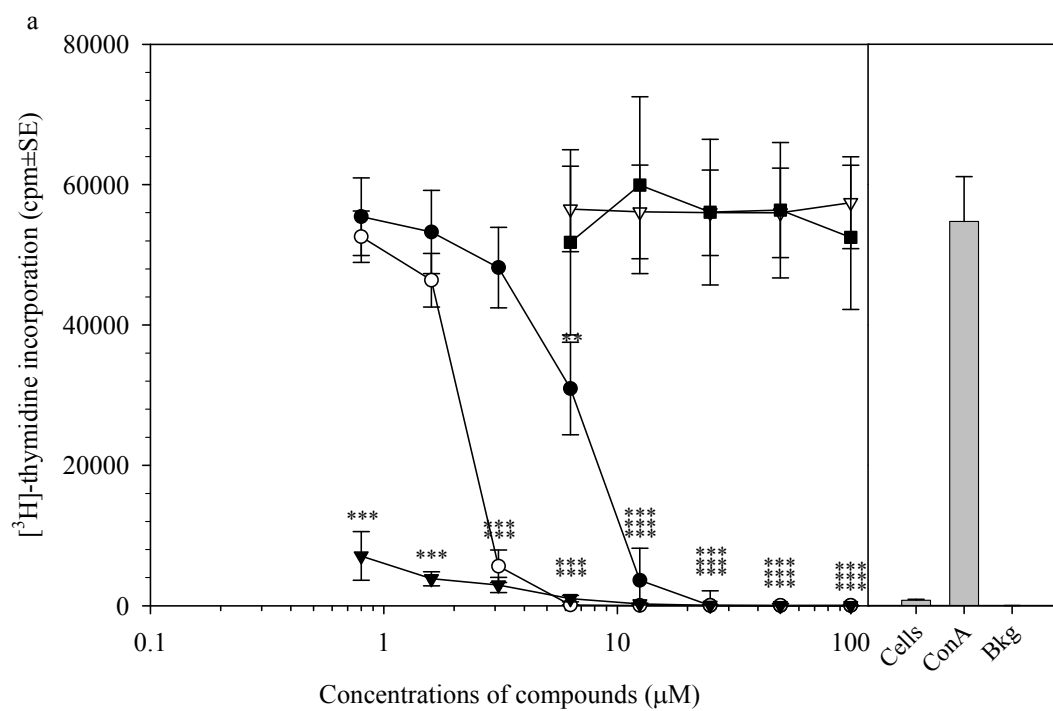
Key: 3-oxo-C12-HSL (n=9); ●, PQS (n=6); ○ , CsA (n=3); ▼ , DMSO (n=9); ▽ , Ethanol (n=3); ■ .
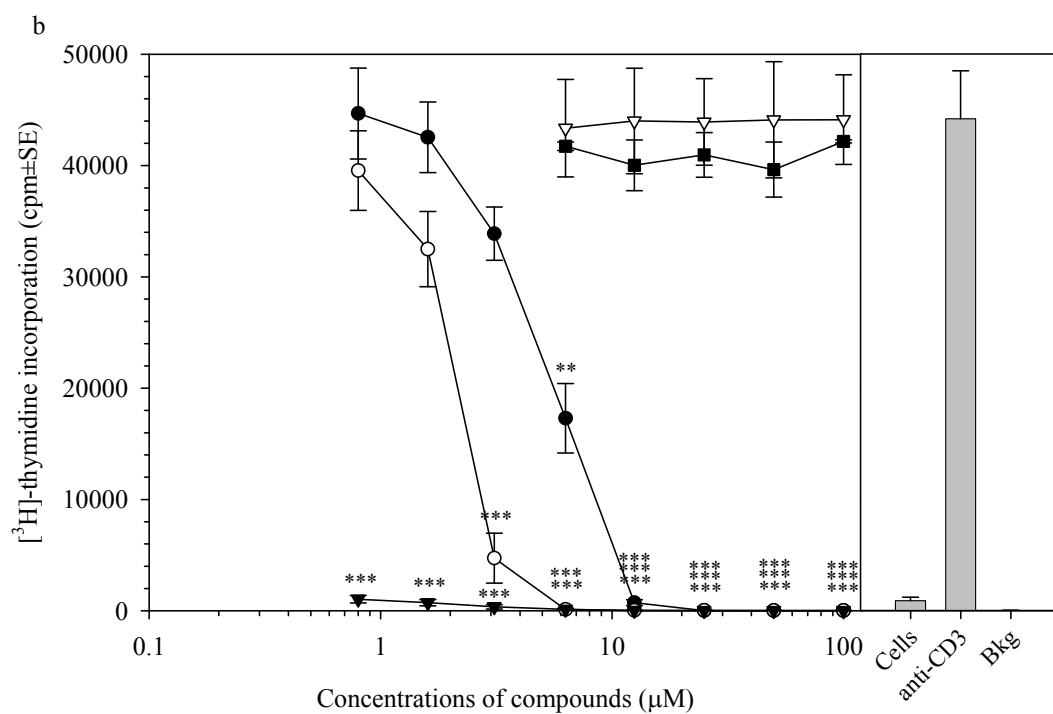
Key: 3-oxo-C12-HSL (n=5); ●, PQS (n=5); ○ , CsA (n=5); ▼ , DMSO (n=5); ▽ , Ethanol (n=3); ■ .

Figure 3.2. The effect of *P. aeruginosa* QSSMs and CsA on ConA (a) and anti-CD3 (b) driven splenocyte proliferation. All three compounds inhibited cell proliferation. (a) PQS gave an IC_{50} of 2.3±0.06 µM compared to 6.3±0.4 µM for 3-oxo-C12-HSL and 2.8±0.7 µM for CsA. (b) Similar to the ConA assay, PQS gave and IC_{50} value of 2.3±0.1 µM compared to 5.7±0.4 µM for 3-oxo-C12-HSL and 2.1±0.3 µM for CsA. DMSO and ethanol were used as diluent controls for the QSSMs and CsA respectively and had no effect on cell proliferation. ***P<0.001 and **P<0.01 compared with the ConA or anti-CD3 control (cells stimulated only with ConA (a) or anti-CD3 (b)). Cell control represents untreated cells. Data represents the mean of independent experiments (n = number of independent experiments); the results for the original experiments are individually plotted in A.2.3 and A.2.4 respectively.

3.3.4. Effect of QSSMs and CsA on murine splenocyte viability

Cytotoxic effects of *P. aeruginosa* QSSMs on mouse splenocytes were assessed before further *in vitro* tests were carried out. Concurrent MTS assay data (fig. 3.3) illustrated that for both 3-oxo-C12-HSL and PQS, suppression of proliferation was evident in the absence of cytotoxicity. Over 92% of cell survival was seen when treated with the selected concentrations of 3-oxo-C12-HSL, over 96% cell survival when treated with the selected concentrations of PQS, and 100% cell survival when treated with the selected concentrations of CsA compared to the untreated cell control.

3.3.5. Cytokine production by spleen cell cultures stimulated with ConA and *P. aeruginosa* QSSMs

To determine whether *P. aeruginosa* QSSMs mediated suppression affects the production of cytokines, the levels of IFNγ and IL-4 in cultures of splenocytes stimulated with ConA alone or in the presence of *P. aeruginosa* QSSMs were determined using the sandwich ELISA technique. ConA stimulation of splenocytes resulted in increased production of IFNγ and IL-4 – 423 pg/ml and 53 pg/ml respectively (fig. 3.4a and fig. 3.4b). Both 3-oxo-C12-HSL and PQS significantly inhibited IFNγ production in a dose dependent manner both having similar potency shown by the IC_{50} value obtained – 3.5 μM and 2.7 μM respectively (fig. 3.4a). 3-oxo-C12-HSL also significantly inhibited IL-4 release in a dose dependent manner with an IC_{50} 8.5 μM (fig. 3.4b) in contrast to PQS, which had no significant effect on IL-4 production except at 100 μM.
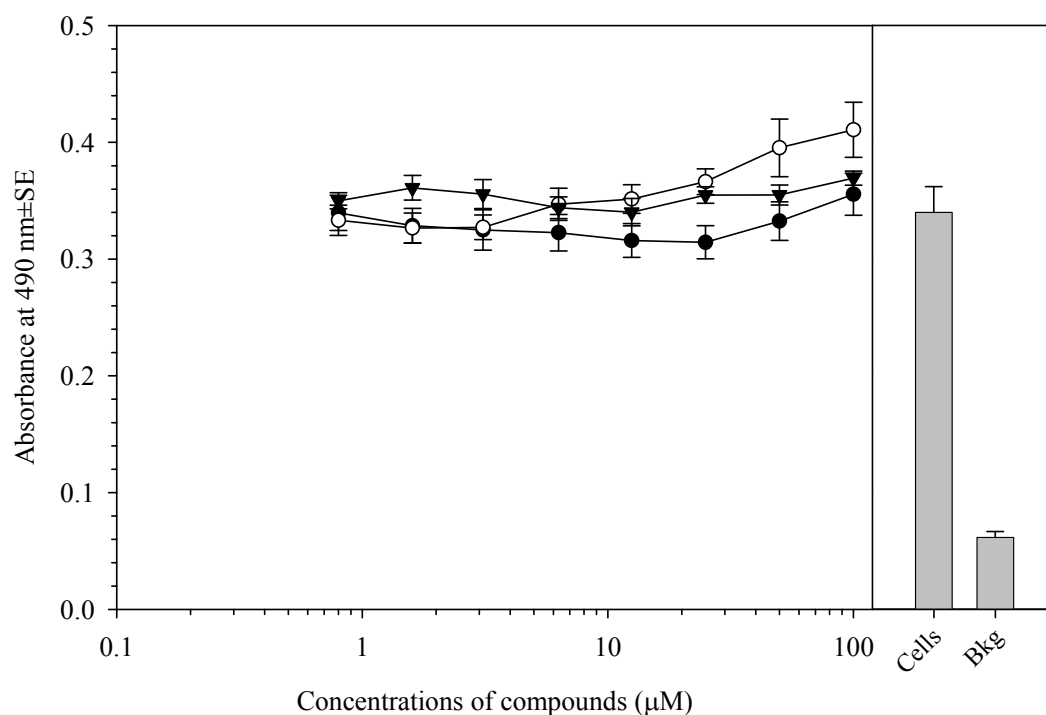
Figure 3.3

Key: 3-oxo-C12-HSL (n=9); ●, PQS (n=9); ○, CsA (n=5); ▼.

Figure 3.3.   The influence of *P. aeruginosa* QSSMs and CsA on murine splenocyte viability as determined by MTS analysis. All three compounds had no cytotoxic effect on murine splenocytes. No significant decrease in absorbance was noted with any of the test groups. DMSO and ethanol were used as diluent controls for the QSSMs and CsA respectively and did not have any effect on cell viability (data not shown).   Cell control represents untreated cells and Bkg (background) represents measurement of CTCM alone. Data represents the mean of independent experiments (*n* = the number of independent experiments); the results of the original experiments are individually plotted in A.2.5.

Figure 3.4

Key: : 3-oxo-C12-HSL; ● , PQS; ○ .
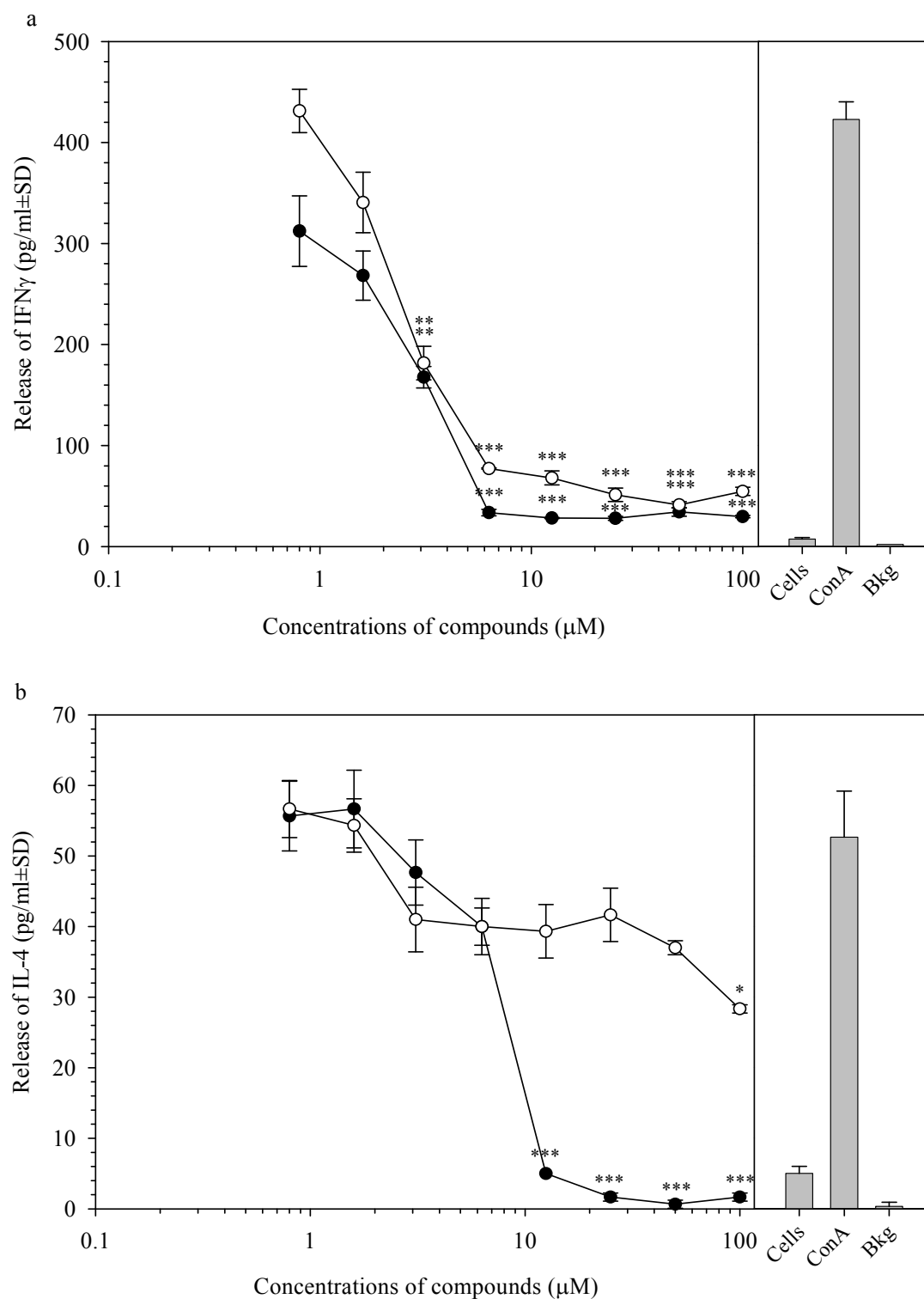
Figure 3.4. Influence of 3-oxo-C12-HSL and PQS on IFNγ (a) and IL-4 (b) production by ConA stimulated murine splenocytes. (a) 3-oxo-C12-HSL and PQS inhibited IFNγ release in a dose dependent manner where PQS has an IC_{50} of 2.7 μM and 3-oxo-C12-HSL has an IC_{50} of 3.5 μM. (b) 3-oxo-C12-HSL inhibited IL-4 release in a dose dependent manner with an IC_{50} of 8.5 μM whereas PQS did not significantly inhibit IL-4 release except at 100 μM. ***$P<0.001$, **$P<0.01$ and *$P<0.05$ compared to ConA control (cells treated with only ConA). Cell control represents untreated cells and Bkg (background) represents reading for CTCM. Results are the mean of triplicate data.

3.3.6. Summary of mouse splenocyte assays

Compounds	IC$_{50}$			
	ConA proliferation	IFNγ production	IL-4 production	αCD3 proliferation
3-oxo-C12-HSL	Inhibition 6.3±0.4 µM	Inhibition 3.5 µM	Inhibition 8.5 µM	Inhibition 5.7±0.4 µM
PQS	Inhibition 2.3±0.06 µM	Inhibition 2.7 µM	Inhibits only at 100 µM	Inhibition 2.3±0.1 µM
CsA	Inhibition 2.8±0.7 µM	Not tested	Not tested	Inhibition 2.1±0.3 µM
DMSO	No effect	No effect	No effect	No effect
Ethanol	No effect	No effect	No effect	No effect

Table 3.1. Summary of the effects of QSSMs and CsA on mouse splenocyte proliferation and cytokine production.

The proliferation assays indicated that all three compounds inhibited splenocyte proliferation. PQS was the more potent anti-proliferative compound shown by the IC$_{50}$ value of 2.3±0.06 µM compared to 6.3±0.4 µM for 3-oxo-C12-HSL in the ConA assay and 2.3±0.1 µM compared to 5.7±0.4 µM in the anti-CD3 proliferation assays. CsA also significantly inhibited ConA and anti-CD3 driven mouse splenocytes with IC$_{50}$ values of 2.8±0.7 µM and 2.1±0.3 µM respectively, similar to the IC$_{50}$ concentrations of PQS. The MTS assays confirm all three compounds did not affect cell viability. Furthermore, the cytokine assays suggest that even though both PQS and 3-oxo-C12-HSL inhibited ConA stimulated splenocyte proliferation, they have differential activity with 3-oxo-C12-HSL inhibiting both IFNγ and IL-4 release while PQS inhibits IFNγ production with no significant effect on IL-4 production.

3.4. Discussion

The aim of this chapter was to investigate the effects of 3-oxo-C12-HSL and PQS on cell proliferation, viability and cytokine release in mouse splenocyte cells. Throughout all the experiments CsA was used as the positive control since its potent immunosuppressive properties are similar to that of 3-oxo-C12-HSL, and so that a comparable analysis could be made to the potency between these three compounds. The concentrations used for the two QSSMs and the incubation time of 48 hr was based on previous studies (Chhabra *et al.*, 2003, Hooi *et al.*, 2004), and allowed both QSSMs to exert their full effects on the cell types used.

3.4.1. Immunosuppressive effects of 3-oxo-C12-HSL and PQS

The initial experiments determined the comparison between 3-oxo-C12-HSL and PQS on BALB/c mice spleen cells, as they were readily available; this allowed for a training element and for reproducible, comparative data to be obtained.

The spleen is formed by reticular and lymphatic tissue and is the largest lymph organ. It was the first choice of organ to use in this study due to its cellular material, consisting of lymphocytes and macrophages (splenic pulp), red pulp which is part suffused with blood and white pulp consisting of areas of lymphatic tissue where there are sleeves of lymphocytes and macrophages around the blood vessels. One of the main functions of the spleen is to bring blood into contact with lymphocytes and to activate lymphocytes if there is foreign antigen contact. The richness of immune cells, such as monocytes, leukocytes and macrophages is advantageous to this study.

The present results confirm that ConA has the ability to induce mitogenic activity of mouse splenocytes in a dose dependent manner (fig. 3.1a). A typical bell shaped course of the ConA dose response curve was obtained representing a proliferative response to suboptimal concentration of ConA and a toxic action of ConA at supraoptimal concentrations. The proliferative and suppressive responses of ConA has been confirmed by Niks et al. (Niks et al., 1990). ConA is one of the most widely used and well-characterised lectins isolated from *Canavalia ensiformis*. ConA has broad applicability primarily because it recognises a commonly occurring sugar structure, α-linked mannose. It is a potent mitogen used to stimulate cell proliferation in lymphocytes and primary T cells independently of accessory cells and activates via the T cell receptor and other cell surface receptors (Powell and Leon, 1970, Novogrod.A and Katchals.E, 1971). ConA has also been shown to activate macrophages and neutrophils (Boldt et al., 1975, Cameron et al., 1983) as well as stimulate toll-like receptor expression (Sodhi et al., 2007).

Fig. 3.2a. clearly demonstrates that 3-oxo-C12-HSL and PQS, which are in hierarchical cascade involved in the regulation of numerous essential *P. aeruginosa* virulence determinants (Passador et al., 1993, Pearson et al., 1994, Diggle et al., 2003), significantly reduced the ability of splenocytes to respond to ConA (IC_{50}, 6.3±0.4 µM and 2.3±0.06 µM, respectively). Although assays involving mitogens do not directly correlate with physiological activities of cells, they do provide a first indication of the pharmacological activity of test compounds. It has been shown that ConA signals via PTK (Asahi et al., 1993), however, 3-oxo-C12-HSL does not inhibit PTK activity (Telford et al., 1998), thus presumably acts at downstream level. Further suggestions on the action of 3-oxo-C12-HSL include possible inhibition of

interaction with the T cell receptors (TCR) or of autophosphorylation. It is equally conceivable that 3-oxo-C12-HSL acts as, or activates a transcription factor in T cells. Optimal activation of human T cells *in vivo* is ensured by the interaction of specific antigen with the T cell receptor (TCR) in the presence of co-stimulatory signals, provided for by antigen-presenting cells (APC).

As the frequency of human antigen-specific T cells in PBMCs samples is very low and autologous or HLA-match APC are rarely available, stimulation of T cells *in vitro* is often carried out with polyclonal activators such as the lectins PHA or ConA, or with the combination of the phorbol ester TPA and calcium ionophore. Although these modes of activation may provide useful information on signalling pathways in T cells, they are, from a biological point of view, artificial and not always comparable to *in vivo* stimulation conditions (Yssel *et al.*, 1992, Byun *et al.*, 1994, Yang *et al.*, 1995b). A more physiological relevant mode of activation is stimulation of T cells with a combination of a monoclonal antibody (mAb) specific for anti-CD3 which mimics antigen-mediated TCR/anti-CD3 complex-mediated signalling (Reinherz *et al.*, 1982), and a anti-CD28 mAb that provides the required co-stimulatory signals (Hara *et al.*, 1985, Geppert and Lipsky, 1987, Schwartz, 1990). Thus to enhance the biological significance of this study, the effect of QSSMs on anti-CD3 driven mouse splenocyte proliferation was examined.

The purified hamster anti-mouse CD3e (CD3ε chain) mAb used reacts with the 25-kDa ε chain of the T cell receptor-associated CD3 complex, which is expressed on thymocytes, mature T-lymphocytes and natural killer T cells. The cytoplasmic domain of anti-CD3 participates in the signal transduction events that activate several

cellular biochemical pathways as a result of antigen recognition. Interestingly, anti-CD28 is not required to stimulate IL-2 release and proliferation when using murine lymphocytes (Green *et al.*, 1994, Shahinian *et al.*, 1993). Many studies have demonstrated that T cells from CD28-deficient mice are still able to produce immune responses to pathogens (Shahinian *et al.*, 1993) and in addition, CD28-deficient T cells can be activated *in vitro* and *in vivo*, albeit at a lower magnitude than normal T cells (Shahinian *et al.*, 1993, Green *et al.*, 1994).

As with ConA, both 3-oxo-C12-HSL and PQS inhibited cell proliferation in a dose dependent manner when splenocytes were stimulated to proliferated using 3 μg/ml anti-CD3. PQS was a consistently more potent anti-proliferative in this assay with an IC_{50} value of 2.3 μM ± 0.1 compared to 5.7±0.4 μM for 3-oxo-C12-HSL (fig. 3.2b) similar to that found in the ConA proliferation assay. In addition, [^{3}H]-thymidine assays indicate CsA inhibited both ConA and anti-CD3 driven splenocyte proliferation with similar potency to PQS – 2.8±0.7 μM and 2.1±0.3 μM respectively (fig. 3.2a and b).

A general observation noted that thymidine uptake was lower when splenocytes were stimulated with anti-CD3 (between 40000 to 50000 cpm) compared to that of ConA (between 55000 to 70000). The difference in cell count may reflect the specificity of anti-CD3 targeting T cells whereas ConA targets a boarder range of receptors.

3.4.2. Effects of 3-oxo-C12-HSL and PQS on IFNγ and IL-4 release from mouse splenocytes

To gain further insight into the molecular mechanisms of action of 3-oxo-C12-HSL and PQS, the effects of these QSSMs on IFNγ and IL-4 production on was investigated.

The effects of 3-oxo-C12-HSL and PQS on IFNγ and IL-4 production were tested as they are markers of T helper 1 (Th1) and T helper 2 (Th2) responses respectively (Mosmann *et al.*, 1986, Cherwinski *et al.*, 1987). Cytokines clearly are important in the development of different cytokine-producing Th1 and Th2 cells. Th1 cells produce several characteristic cytokines, most notably IL-12 and IFNγ, whereas Th2 cells produce a set of cytokines, most notably IL-4, IL-5 and IL-13. In turn, IL-2 and IFNγ promote the development of strong cell-mediated immunity, whereas the type 2 cytokines promote allergic responses effective in eliminating parasites (Kuo and Leiden, 1999, Glimcher and Murphy, 2000, Murphy *et al.*, 2000). Although not absolute, one of the most critical elements of determining Th differentiation is the cytokine milieu in which the T cell is activated.

Both 3-oxo-C12-HSL and PQS inhibited production of IFNγ in ConA stimulated murine leukocytes in a dose dependent manner (fig. 3.4a). IFNγ production inhibited in the presence of 3-oxo-C12-HSL and PQS suggest that it is unlikely that they are activating NFκB-dependent pathways in T cells since such activation is associated with increased transcription and production of inflammatory mediators such as IFNγ (Sica *et al.*, 1992, Sica *et al.*, 1997). The inhibition of IFNγ by both QSSMs suggest

suppression of T cell activation is via Th1 as IFNγ is required for the promotion of the Th1 linage. This supports Telford *et al.*'s study in which the authors demonstrated reduction of total IL-12 production by LPS-stimulated macrophages, as cytokine that directs the generation of a Th1 response via the induction of IFNγ secretion (Telford *et al.*, 1998). 3-oxo-C12-HSL also inhibited IL-4 release while PQS had no significant effect on IL-4 production in mouse splenocytes (fig. 3.4b). IL-4 is required to stimulate a Th2 response. The inhibition of IL-4 as well as IFNγ by 3-oxo-C12-HSL suggests this QSSM may suppress T cell response to *P. aeruginosa* infections by inhibiting both Th1 and Th2 activity, or is a less specific inhibitor of T cell function. Ritchie *et al.* also demonstrated *in vitro* exposure of mouse spleen cells to 3-oxo-C12-HSL had suppressive effects on both IFNγ and IL-4 release, however, their *in vivo* study demonstrated an increase in IFNγ and IL-4 production (Ritchie *et al.*, 2003). This highlights important implications for extrapolating *in vitro* results to models of *P. aeruginosa* infections.

To conclude, results obtained in this study demonstrates that 3-oxo-C12-HSL and PQS exhibit immune suppressive properties in mouse splenocytes, and suggests this may be due to suppression of the Th1 pathway – a pathway that is important to the clearance of bacteria. To further understand the effects of *P. aeruginosa* quorum sensing signalling molecules on human immune cells, this study move on to using human peripheral mononuclear cells and pure human T cells isolated from venous whole blood, to obtain more biologically relevant data.

4. Assessment of the effect 3-oxo-C12-HSL, PQS and CsA on hPBMC and pure human T cell proliferation and cytokine production

4.1. Introduction

As demonstrated in Chapter 3, 3-oxo-C12-HSL and PQS are immune-suppressive with indication that PQS inhibits via the Th1 pathway, while 3-oxo-C12-HSL may inhibit both Th1 and Th2 pathways, or is a less specific inhibitor of T cell function. The aim of this chapter is to look at the effects of these two QSSMs and CsA on more biologically relevant cell types – first on human PBMC and finally on pure human T cells. At each stage, the effect on cell proliferation, viability and cytokine release was assessed.

Hooi *et al.* demonstrated the immune suppressive properties of 3-oxo-C12-HSL and PQS on anti-CD3 and anti-CD28 driven human PBMCs. In addition, it was also shown 3-oxo-C12-HSL inhibited TNFα release from human PBMCs as also shown by Telford *et al.* in mouse peritoneal macrophages (Telford *et al.*, 1998), and inhibited IL-2 production. In contrast, PQS induced the release of both TNFα and IL-2 (Hooi *et al.*, 2004), highlighting the differential immune-modulatory activity of these two QSSMs. This differential activity lead the authors to suggest 3-oxo-C12-HSL may inhibit T cell activity by suppressing upstream of the IL-2 signalling pathway, while PQS may inhibit downstream of the IL-2 signalling pathway, actions reminiscent of the immune-suppressive drugs CsA and rapamycin respectively.

4.2. Methods

To understand the effects of 3-oxo-C12-HSL and PQS on human PBMC and pure T cells, cell proliferation, viability and cytokine production were examined.

- The effects of 3-oxo-C12-HSL and PQS on hPBMC proliferation using [^{3}H]-thymidine incorporation, viability using MTS-PMS analysis and IL-2 production using ELISA (chapter 4.2.1).

- The effects of 3-oxo-C12-HSL and PQS on pure human T cell proliferation using [^{3}H]-thymidine incorporation and viability using MTS-PMS analysis (chapter 4.2.2).

- The effects of 3-oxo-C12-HSL and PQS on IL-2 production using ELISA and IL-2, IL-4, IL-5, IL-10, IFNγ and TNFα production using cytometric bead analysis (CBA) in pure T cells (chapter 4.2.3 and section 4.2.4 respectively).

4.2.1. Human Peripheral blood mononuclear cells

4.2.1.1. hPBMC isolation and stimulation

Blood specimens were obtained with consent from healthy human volunteers' age ranging from 22 to 45 years. Human PBMCs were isolated from heparinised (100 unit/ml) venous whole blood by buoyant density centrifugation over histopaque 1077 (Sigma, Poole, UK). Briefly, blood was diluted to a ratio of 1:1 with CTCM and layered gently onto the surface of histopaque 1077 (at a ratio of 1:2 respectively) before centrifuging at 450 x g for 20 min at RT. The upper plasma layer was

discarded and using a sterile pipette, the cloudy layer containing the PBMC was gently collected and placed in a sterile 20 ml universal tube. A further 10 ml CTCM was added into the PBMC suspension and centrifuged at 600 x *g* for 10 min before repeating the wash. Finally, the pellet was re-suspended in 1 ml CTCM. The concentration of anti-CD3 and anti-CD28 that induced optimal PBMCs proliferation was determined in preliminary dose response experiments with concentration ranging 0.003 μg/ml to 0.4 μg/ml and 0.15 μg/ml to 20 μg/ml respectively.

Cell density was determined using the trypan blue assay and adjusted to 10^6 cells/ml. 100 μl cell suspension was added per well of 96-well round-bottom tissue culture plates. PBMCs were stimulated with anti-CD3 monoclonal antibody (mAb) (0.1 μg/ml) and anti-CD28 mAb (5 μg/ml), and further stimulated with either 3-oxo-C12-HSL, PQS and CsA (100 μM and subsequent half dilutions). PBMCs were cultured for 24 hr at 37^oC in 5% CO_2. Control cells were performed, stimulating PBMCs with only αCD3 and αCD28 or resting PBMCs (non-stimulated). 100 μl of cell culture supernatants was removed and stored at -20^oC until required for cytokine analysis after 24 hr. Cells were then incubated for a further 24 hr at 37^oC.

4.2.1.2. Proliferation assay

After 24 hr, cells were pulsed with 10 μl per well (0.25 μCi) ^{3}H-thymidine (^{3}H-LThy; Amersham, UK) and incubated for 24 hr at 37^oC in 5% CO_2. Finally, cultures were harvested on glass-fibre culture (GF/C) plates (Unifilter Filtermate Harvester^{TM}, Packard Biosciences Ltd, UK), left to dry at 37^oC for 2 hr, followed by the addition of 40 μl scintillant (MicroScint-O^{TM}, Packard Biosciences Ltd, UK) to

each well. Incorporated radioactivity was measured in a liquid scintillation β-counter (Topcount[TM], Packard Bioscience Ltd, UK) according to manufacturers' instructions. Results are expressed as mean counts per minute (cpm) of triplicate wells ± SD (Appendix 2) and mean cpm of all individual data ± SE (main chapter). Statistical analysis was performed applying the parametric, ordinary ANOVA test.

4.2.2. Human T cells

4.2.2.1. T cell isolation

hPBMC cells were isolated as described previously (section 4.2.1.1). 10^7 cells were re-suspended in 10 ml CTCM and centrifuged at 600 x g for 10 min. Pure human T cells were then isolated using the negative selection Human Pan T cell Isolation Kit II (Miltenyi Biotec Inc., Surrey, UK) with biotin-conjugated monoclonal antibodies against CD14, CD16, CD19, CD36, CD56, CD123 and glycophorin A.

Briefly, after centrifugation of PBMC cells, supernatant was removed completely and re-suspended in 40 μl of degassed T cell isolation buffer (PBS pH 7.2 supplemented with 0.5% BSA and 2 mM EDTA), 10 μl biotin-antibody cocktail was added and mixed well by swinging the universal tube from side to side before incubating at 4^oC for 10 min. A further 30 μl of T cell isolation buffer and 20 μl anti-biotin microbeads was added, mixing well before incubating at 4^oC for 15 min. Cells were then washed with T cell isolation buffer by adding 10-20 times the labelling volume and centrifuging at 300 x g for 10 min at 4^oC. The supernatant was completely removed before re-suspending in 500 μl of T cell isolation buffer.

The magnetically labelled non-T cells are depleted by retaining them on a MACs LS column (Miltenyi Biotec Inc., Surrey, UK) in the magnetic field of a MACs Separator (Miltenyi Biotec Inc., Surrey, UK), while unlabelled T cells pass through the column and are collected. The MACs LS column was placed in the magnetic field of a miniMACs separator. The column was prepared the column by rinsing with 3 ml of T cell isolation buffer. The cell suspension was then pipetted into the column, allowing the cells to pass through completely, collecting the enriched T cell fraction into a universal tube. A further 3 ml of T cell isolation buffer was added 3 times to the column, collecting the entire effluent representing the enriched T cells into the same universal tube. 10 ml of CTCM was added to the enriched T cell suspension and centrifuged at 600 x g for 10 min, discarding the supernatant and re-suspended in 1 ml CTCM. Cell density was determined using trypan blue assay.

4.2.2.2. Evaluation of T cell purity using flow cytometry

T cells were isolated and their density determined using trypan blue assay. 10^5 total cells in CTCM were centrifuged at 300 x g for 10 min and supernatant removed completely before re-suspending into test tubes using 15 ml T cell isolation buffer and centrifuging at 600 x g for 10 min. The supernatant was removed completely and cell pellet re-suspended in 1 ml 0.5% formaldehyde in saline and incubating at room temperature for 30 min. The suspension was then centrifuged at 600 x g for 10 min, discarding the supernatant before washing twice with PBS supplemented with 2% (*w/v*) FCS and 0.1% (*w/v*) sodium azide (PBA) and centrifuging at 600 x g for 10 min. The supernatant was discarded and pellet re-suspended in the residue. Fluorescence-labelled antibody - CD25-PE (phycoerythrin) 5 µl/tube was added to

detect T cell activation and CD3-ECD (an energy transfer fluorophore consisting of Texas red coupled to PE) 5 μl/tube was added to detect T cell purity (DAKO, Trappes, France) - and incubated for 45 min away from light. A further 1 ml of PBA was added to the T cells and centrifuged at 600 x g for 10 min. Finally, the treated T cells were re-suspended in 0.5 ml PBA and fluorescence was detected using fluorescence-activated cell sorter (FACSCalibur; BD Biosciences, UK) and analysed using WinMDI program.

4.2.2.3. T cell stimulation

The concentration of anti-CD3 and anti-CD28 that induced optimal T cell proliferation was determined in preliminary dose response experiments with concentration ranging 0.003 μg/ml to 0.4 μg/ml and 0.15 μg/ml to 20 μg/ml respectively. T cells were isolated and cell density determined using the trypan blue assay. 100 μl cell suspension at 10^6 cells/ml in CTCM was added per well of 96-well round-bottom tissue culture plates. 3-oxo-C12-HSL, PQS and CsA were tested at final concentrations of 100 μM and subsequent half dilutions and cells were stimulated with 0.1 μl/ml anti-CD3 and 5 μg/ml anti-CD28. T cells were cultured for 24 hr at 37°C in 5% CO_2. Control cells were performed, stimulating T cells with only anti-CD3 and anti-CD28 or resting T cells (non-stimulated). After 24 hr, 100 μl of cell culture supernatant was removed and stored at -20°C for the cytokine assays, and cells incubated for a further 24 hr at 37°C.

4.2.2.4. Proliferation assay

After 24 hr, cells were pulsed with 10 µl 0.25 µCi/well [^{3}H]-thymidine (^{3}H-LThy; Amersham, UK) and incubated for 24 hr at 37°C in 5% CO_2. Finally, cultures were harvested on glass-fibre culture (GF/C) plates (Unifilter Filtermate Harvester^{TM}, Packard Biosciences Ltd, UK), left to dry at 37°C for 2 hr, followed by the addition of 40 µl scintillant (MicroScint-O^{TM}, Packard Biosciences Ltd, UK) to each well. Incorporated radioactivity was measured in a liquid scintillation β-counter (Topcount^{TM}, Packard Bioscience Ltd, UK) according to manufacturers' instructions. Results are expressed as mean counts per minute (cpm) of triplicate wells ± SD (Appendix 2) and mean cpm of all individual data ± SE (main chapter). .

4.2.2.5. Cytokine determination using Cytometric Bead Array

Flow cytometry is an analysis tool that allows for the discrimination of different particles based on size and colour. Multiplexing is the simultaneous assay of many analytes in a single sample. The BD^{TM} CBA (BD biosciences, Europe) was chosen for this assay. BD^{TM} CBA employs a series of particles with discrete fluorescence intensities to simultaneously detect multiple soluble analytes, and when combined with flow cytometry, a multiplexed assay is created. The BD^{TM} CBA Human Th1/Th2 Cytokine Kit (BD biosciences, Europe) was used to quantitatively measure IL-2, IL-4, IL-5, IL-10, TNF and IFNγ protein levels in a single sample. Alterations to the protocol were made and were used to supplement the manufacturer's protocol.

Firstly, the human Th1/Th2 cytokine standards were prepared. One vial of lyophilized human Th1/Th2 cytokine standards was reconstituted with 200 µl of assay diluents (provided in kit) to prepare a 10x bulk standard. The reconstituted standard was allowed to equilibrate for 20 min before agitating the vial to mix thoroughly. The 10x bulk standard was aliquoted to 10 µl eppendorfs and stored at -20°C. 12 x 75 mm FACs tubes (BD Biosciences, Europe) were labelled and arranged in the following order: Top Standard, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256. 90 µl of assay diluents was added to the top standard tube while 30 µl of assay diluents was added to each of the remaining tube. 10 µl of 10x bulk standard was then transferred to the top standard tube and mixed thoroughly by inverting tube. Serial dilutions followed by transferring 30 µl from the top standard to the 1:2 dilution tube and mixed thoroughly before transferring 30 µl from the 1:2 tube to 1:4 tube and so on to the 1:256 tube mixing thoroughly each time. The assay diluents reagent served as the negative control.

Next, mixed human Th1/Th2 cytokine capture beads were prepared. The capture beads are bottled individually (i.e. each cytokine in a different bottle) and needed to be pooled together immediately before use. The number of assay tubes including standards and controls was determined and labelled. The capture bead suspension was vigorously vortexed for a few seconds before use. 3 µl aliquot of each capture bead for each assay tube to be analysed was added to a single tube labelled 'mixed capture beads' and vortexed to mix thoroughly. 15 µl of the mixed capture beads was added to the appropriate assay tubes followed by 15 µl of the human Th1/Th2 PE detection reagent (included in the kit). 15 µl of the human Th1/Th2 cytokine standard dilutions was then added to the control assay tubes while 15 µl of T cell

supernatant test samples was added to the test assay tubes. The assay tubes were incubated for 3 hr at room temperature and protected from direct exposure to light before washing with 500 µl of wash buffer (included in kit) and centrifuged at 200 x g for 5 min. The supernatant was carefully aspirated and discarded from each assay tube and the bead pellets re-suspended in 300 µl of wash buffer before reading on a flow cytometer. Results were analysed using the BD CBA software (BD biosciences, Europe).

4.3. Results

4.3.1. Human PBMCs

4.3.1.1. Optimisation of assay condition

The dose response of anti-CD3 and anti-CD28 were initially assessed stimulating human PBMC with 0.4 μg/ml anti-CD3 and subsequent half dilutions with 20 μg/ml anti-CD28 and subsequent half dilutions. Results indicated anti-CD3 and anti-CD28 stimulated cellular proliferation in a dose dependant manner. The optimal dose for anti-CD3 and anti-CD28 is 0.1 μg/ml and 5 μg/ml respectively (fig. 4.1). The optimal dose of anti-CD3 and anti-CD28 was used to stimulate cell proliferation to test the effects of *P. aeruginosa* QSSMs on hPBMC cells.

4.3.1.2. Effects of QSSMs and CsA on hPBMC proliferation

3-oxo-C12-HSL and PQS inhibited human PBMC proliferation in a dose dependent manner when stimulated with 0.1 μg/ml anti-CD3 and 5 μg/ml anti-CD28 antibodies (fig. 4.2). PQS gave an IC_{50} value of 3.5±0.34 μM compared to 42.4±4 μM for 3-oxo-C12-HSL. CsA also suppressed cell proliferation with an IC_{50} value of 5.1±0.35 μM. DMSO and ethanol were used as diluent controls to the QSSMs and CsA respectively and had no significant effect on cell proliferation.

4.3.1.3. Effects of QSSMs and CsA on hPBMC viability

PQS and CsA had no significant cytotoxic effect on human PBMC viability (fig. 4.3). Over 97% of cells survived when treated with the selected concentrations of PQS, and over 80% of cells survived when treated with the selected concentrations of CsA. Over 97% of cells survived when treated with ≤ 12.5 μM 3-oxo-C12-HSL. 83% of cells survived when treated with 25 μM 3-oxo-C12-HSL, and less than 65% of cells survived when treated with ≥ 50 μM 3-oxo-C12-HSL.

4.3.1.4. IL-2 production by human PBMC cell cultures stimulated with anti-CD3 and anti-CD28 and *P. aeruginosa* QSSMs or CsA

To determine whether *P. aeruginosa* QSSMs mediated hPBMC suppression affects IL-2 production, the levels of IL-2 in cultures of hPBMC stimulated with anti-CD3 and anti-CD28 alone or in the presence of QSSMs was assessed by ELISA. Anti-CD3 and anti-CD28 stimulation of hPBMCs resulted in increased IL-2 release - 1210±125 pg/ml compared to untreated cell control (8.3±1.2 pg/ml) (fig. 4.4). 3-oxo-C12-HSL inhibited the release of IL-2 giving an IC_{50} value of 18±4 μM while PQS had no significant effect on Il-2 production. The presence of IL-2 was also seen in the CsA assay giving levels of IL-2 present similar to the anti-CD3/28 control (cells treated with anti-CD3 and anti-CD28 only) (fig. 4.4).
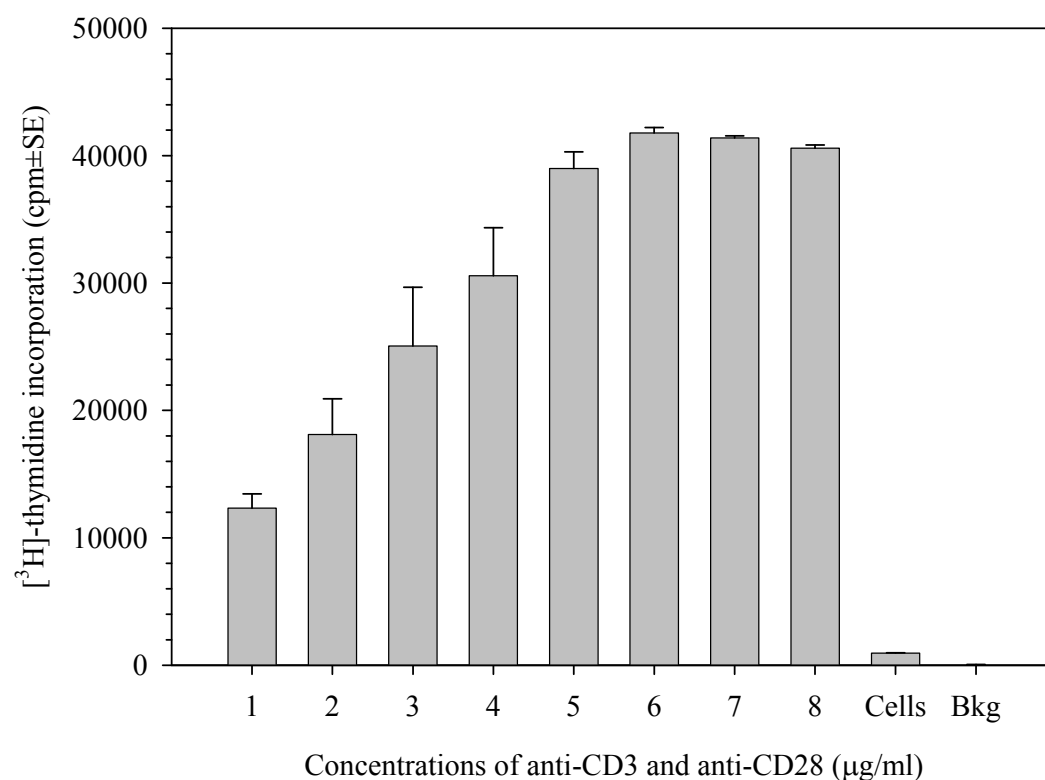
Figure 4.1

Figure 4.1. Stimulatory effect of anti-CD3 and anti-CD28 on human PBMC proliferation. Anti-CD3 and anti-CD28 stimulated cellular proliferation in a dose dependent manner with an optimal dose of 0.1 µg/ml and 5 µg/ml respectively. 1 = 0.003/0.15 µg/ml, 2 = 0.006/0.312 µg/ml, 3 = 0.012/0.62 µg/ml, 4 = 0.025/1.25 µg/ml, 5 = 0.05/2.5 µg/ml, 6 = 0.1/5 µg/ml, 7 = 0.2/10 µg/ml and 8 = 0.4/20 µg/ml of anti-CD3/28. Data represents the mean of 4 independent experiments; results of the original experiments are individually plotted in A.2.6.

Figure 4.2
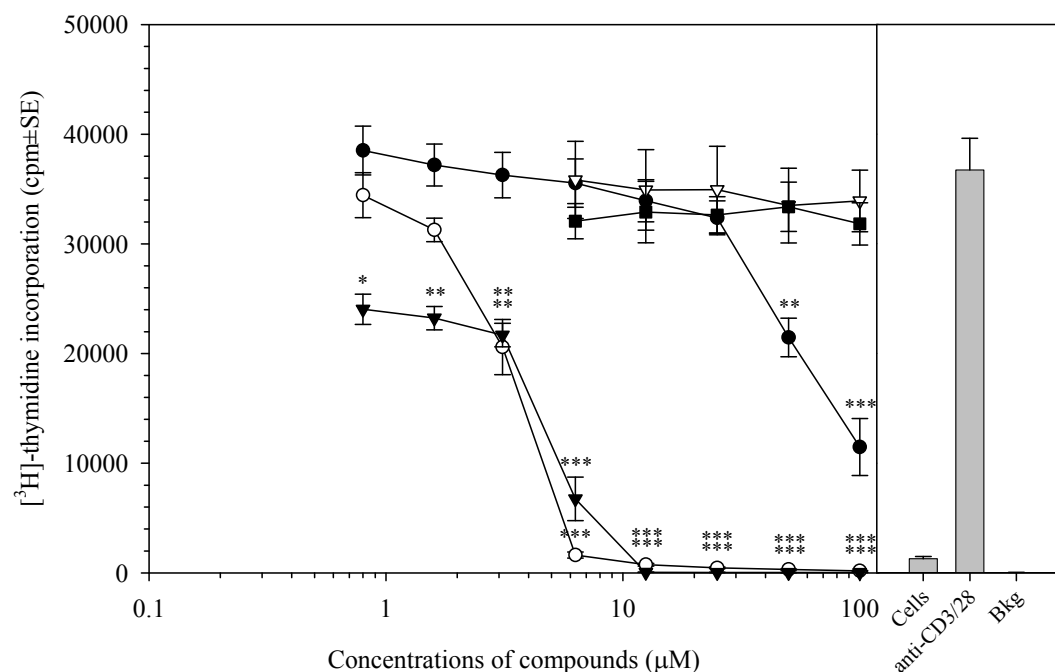
Key: 3-oxo-C12-HSL (n=5); ●, PQS (n=5); ○, CsA (n=5); ▼, DMSO (n=4); ▽, Ethanol (n=4); ■ .

Figure 4.2. The effect of *P. aeruginosa* QSSMs and CsA on anti-CD3 and anti-CD28 driven hPBMC proliferation. 3-oxo-C12-HSL, PQS and CsA inhibited cell proliferation. DMSO and ethanol were used as diluents controls for the QSSMs and CsA respectively and had no effect on cell proliferation. ***P<0.001, **P<0.01 and *P<0.05 compared with the anti-CD3/28 control. Cells control represents untreated cells. Data represents the mean of independent experiments (n = number of independent experiments); the results of the original experiments are individually plotted in A.2.7.

Figure 4.3
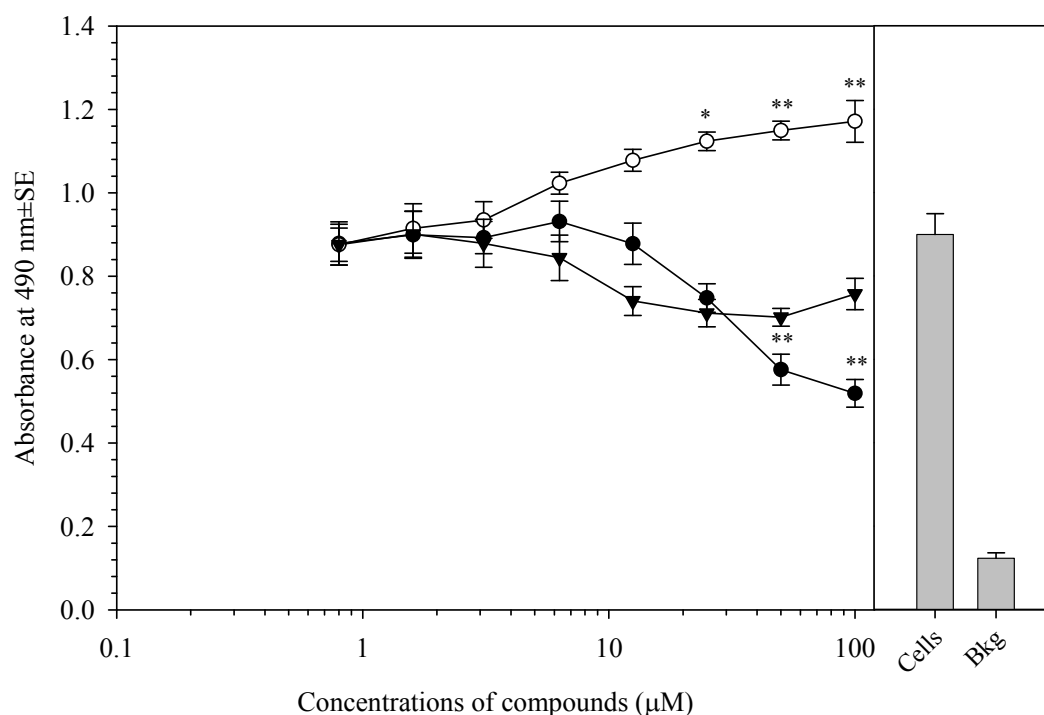
Key: 3-oxo-C12-HSL; ● , PQS; ○ , CsA; ▼ .

Figure 4.3. The effect of *P. aeruginosa* QSSMs and CsA on hPBMC viability. 3-oxo-C12-HSL inhibited cell proliferation before cell viability was affected while with PQS and CsA, immune suppression was evident in the absence of cytotoxicity. **P<0.01 compared to the untreated cell control. Data represents the mean of 6 independent experiments; the results of the original experiments are individually plotted in A.2.8.

Figure 4.4
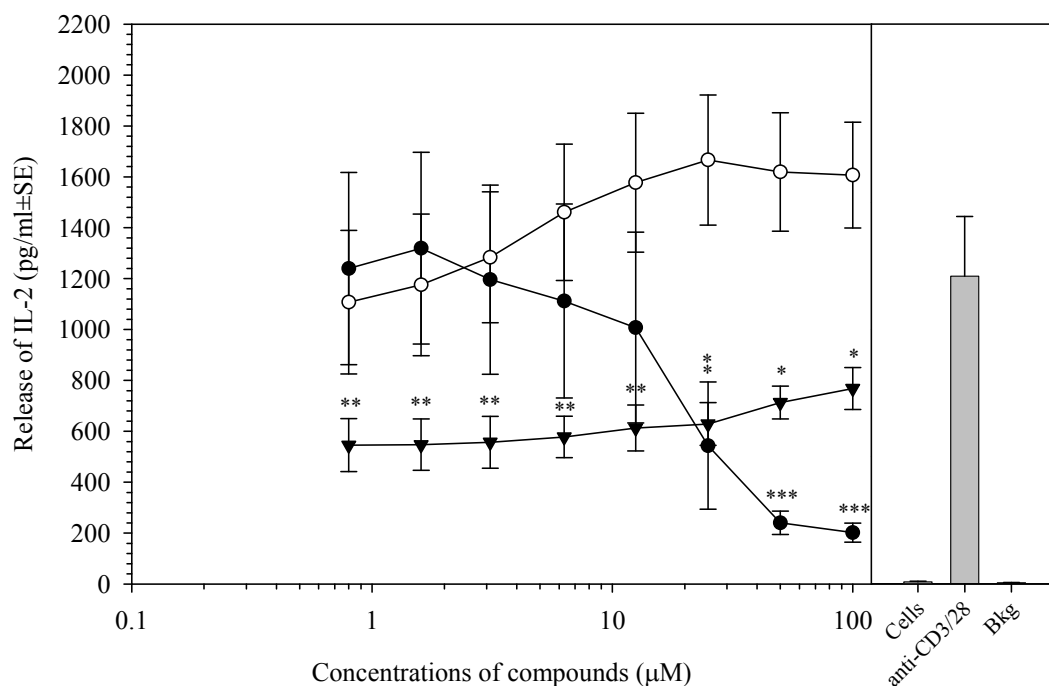
Key: 3-oxo-C12-HSL; ●, PQS; ○, CsA; ▼,

Figure 4.4. The effect of *P. aeruginosa* QSSMs and CsA on the release of IL-2 from hPBMC cells determined by ELISA. 3-oxo-C12-HSL inhibited IL-2 release whereas PQS had no significant effect on the release of IL-2. CsA also significantly inhibited IL-2 release. ***$P<0.001$, **$P<0.01$, *$P<0.05$ compared to the anti-CD3/28 control (cells treated only with antibodies). Cell control represents untreated cells and Bkg (background) represents readings for CTCM. Data represents the mean of 5 independent experiments; the results of the original experiments are individually plotted in A.2.9.

4.3.1.5. Summary of human PBMC assays

Compounds	IC$_{50}$	
	Anti-CD3 and anti-CD28 proliferation	IL-2 production
3-oxo-C12-HSL	Inhibition 42.4±4 μM	Inhibition 18±4 μM
PQS	Inhibition 3.5±0.34 μM	No significant effect
CsA	Inhibition 5.1±0.35 μM	Inhibits at all concentration
DMSO	No significant effect	No significant effect
Ethanol	No significant effect	No significant effect

Table 4.1. Summary of the effects of QSSMs and CsA on hPBMC proliferation and cytokine production.

The establishment of reproducible data for the effects of QSSMs on human leukocytes in culture is seen. PQS inhibits proliferation of hPBMC without affecting cell viability, while 3-oxo-C12-HSL is also immunosuppressive to PBMCs with an immunosuppressive window of 28.5±2.1 μM. At high concentrations, 3-oxo-C12-HSL causes cell death. Cytokine assays confirm the differential activity of both QSSMs. This allowed progression to looking at the effects of these two QSSMs on purified T cells.

4.3.2. Pure human T cell

4.3.2.1. Evaluation of the purity and activity of isolated human T cells

FACs analysis was used to detect the percentage of pure T cells isolated and their state of activation. Fig. 4.5a shows inactive hPBMC cell mixture, while fig. 4.5b indicates 93% of inactive pure T cells were present.

4.3.2.2. Pure human T cell proliferation following stimulation with anti-CD3 and anti-CD28 and *P. aeruginosa* QSSMs or CsA

3-oxo-C12-HSL and PQS inhibited T cell proliferation in a dose dependent manner when stimulated to proliferate with 0.1 μg/ml anti-CD3 and 5 μg/ml anti-CD28 antibodies (fig. 4.6a). PQS and 3-oxo-C12-HSL have IC_{50} values of 3.8±0.15 μM and 19±1.62 μM respectively. The positive control, CsA was also found to inhibit proliferation with an IC_{50} value of 3.2±0.31 μM.

4.3.2.3. Effects of QSSMs and CsA on T cell viability

The cytotoxic effects of these *P. aeruginosa* QSSMs on human T cells was also tested using the MTS assay (fig. 4.6b). Results indicate PQS and CsA did not affect T cell viability at the concentrations and incubation times tested (over 96% and 95% cell survival respectively). In contrast, 3-oxo-C12-HSL did not affect cell viability at low concentrations, but caused cell death at high concentrations. 90% of cells survived when treated with ≤ 12 μM 3-oxo-C12-HSL, 85% cells survived when

treated with 25 μM 3-oxo-C12-HSL, and fewer than 76% of cells survived when treated with ≥ 50 μM 3-oxo-C12-HSL.

Figure 4.5

a

Inactive hPBMC cell mixture

b

93% inactive pure human T cell

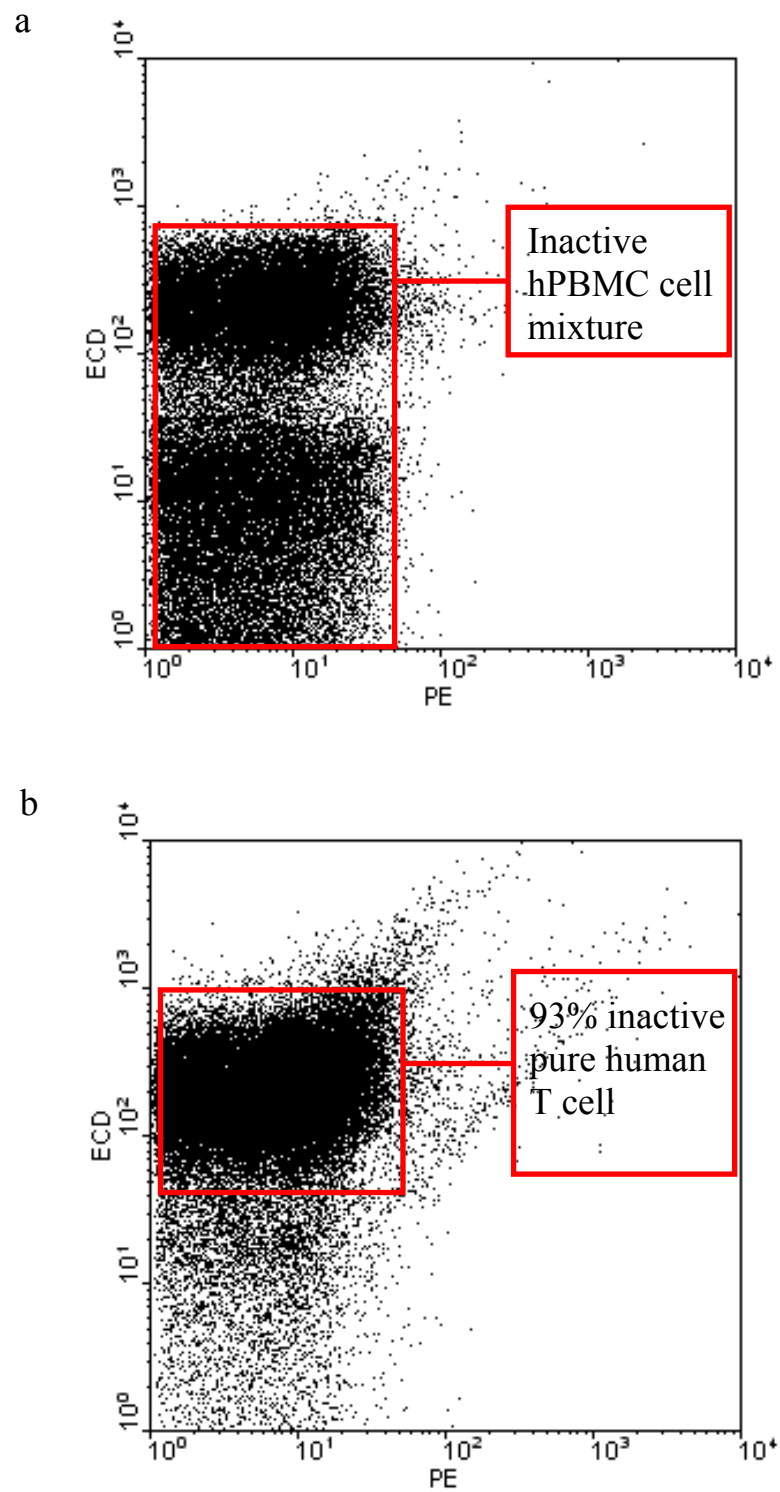**Figure 4.5. Assessment of T cell activation and purity using FACs analysis.**

Total PBMC cells (a) and T cells (b) were isolated and treated with CD25-PE to detect cell activation and CD3-ECD to detect T cell purity. Graph (a) indicates human PBMC cell mixture while graph (b) shows the proportion of pure human T cells isolated. hPBMCs and T cells were isolated in their inactive form. Purified T cells were 93% pure.

Figure 4.6
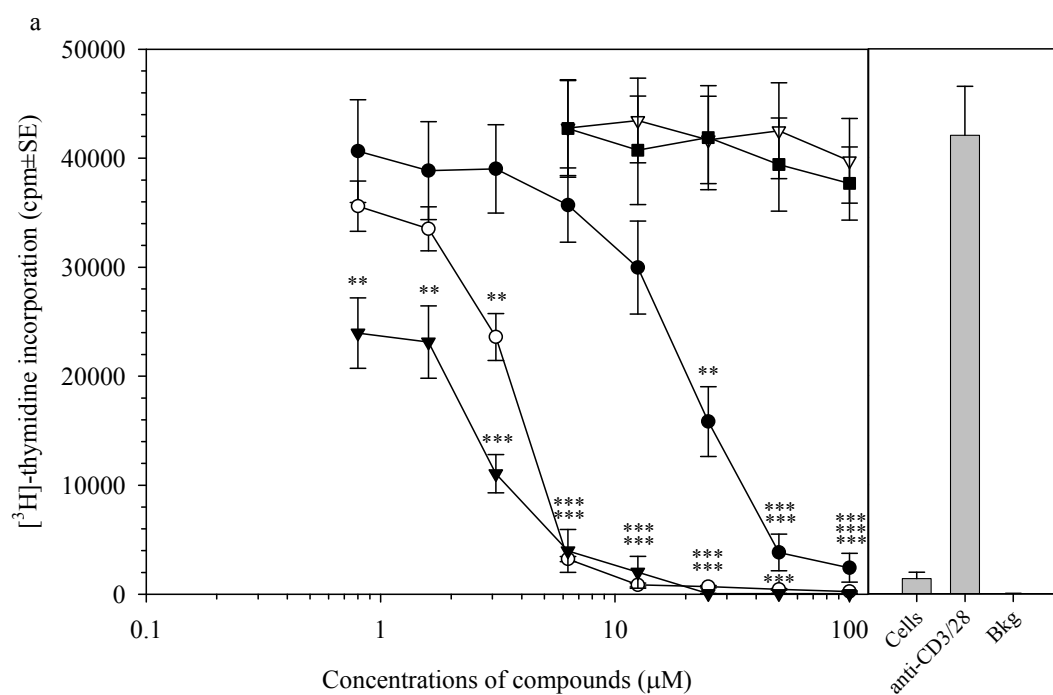
Key: 3-oxo-C12-HSL; ● , PQS; ○ , CsA; ▼ , DMSO; ▽ , Ethanol; ■ .
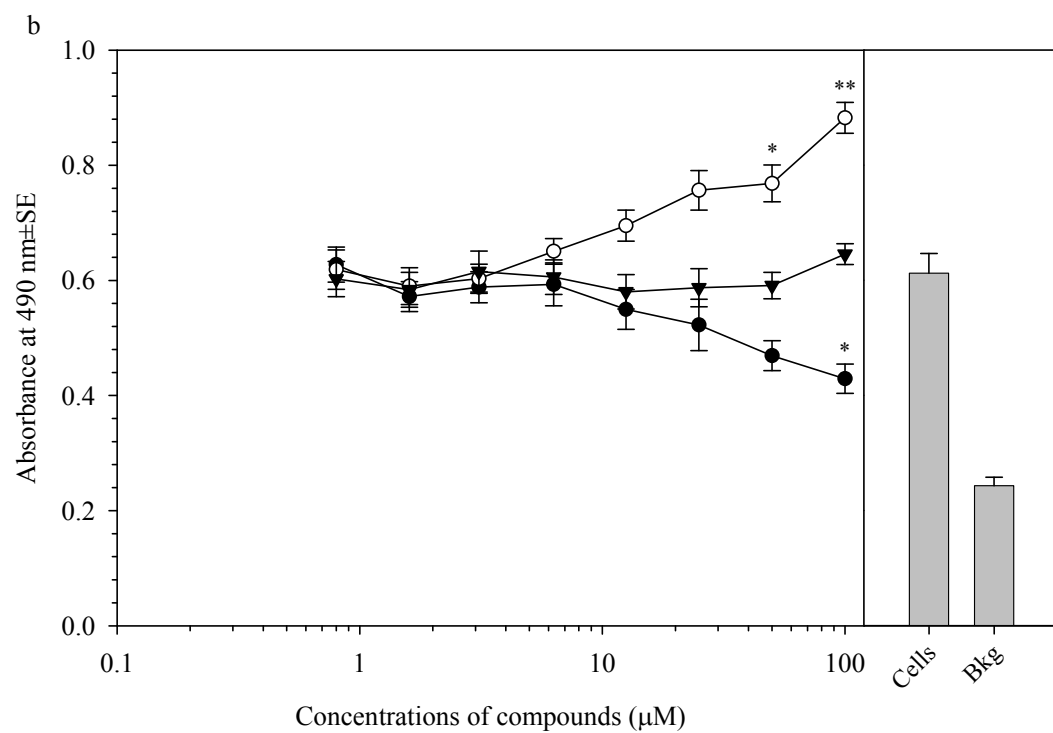
Key: 3-oxo-C12-HSL; ● , PQS; ○ , CsA; ▼ .

Figure 4.6. The effect of *P. aeruginosa* QSSMs and CsA on anti-CD3 and anti-CD28 driven pure human T cell proliferation (a) and viability (b). (a) 3-oxo-C12-HSL, PQS and CsA inhibited T cell proliferation. (b) 3-oxo-C12-HSL inhibited cell proliferation before cell viability was affected while with PQS and CsA, immune suppression was evident in the absence of cytotoxicity. DMSO and ethanol were used as diluent controls for the QSSMs and CsA respectively and had no effect on cell proliferation nor were they cytotoxic to the T cells. ***P<0.001, **P<0.01 and *P<0.05 compared with the anti-CD3/28 control (cells treated with anti-CD3 and anti-CD28 only). Data represents the mean of 7 independent experiments for the proliferative assay and 6 independent experiments for the viability assay; the results of the original experiments are individually plotted in A.2.10 and A.2.11 respectively.

4.3.2.4. Cytokine production by pure human T cell cultures

To determine the effect of *P. aeruginosa* QSSMs' mediated T cell suppression on the production of IL-2, the levels of IL-2 in cultures of T cells stimulated with anti-CD3 and anti-CD28 alone or in the presence of 3-oxo-C12-HSL or PQS was assessed by ELISA and CBA. Anti-CD3 and anti-CD28 stimulation of T cells resulted in increased production of IL-2 – 2480 ± 200 pg/ml compared to untreated cell control of 52 ± 10 pg/ml for the ELISA assay (fig. 4.7a), and this was further confirmed with the CBA assays (1925 ± 105 pg/ml compared to the untreated cell control of 41 ± 19 pg/ml, fig. 4.7b). 3-oxo-C12-HSL inhibited IL-2 release giving an IC_{50} value of 15.7 ± 4.1 μM using the ELISA technique and 28.5 ± 0.5 μM using the CBA kit, while PQS significantly enhance IL-2 production above 25 μM for both the ELISA assay and CBA assay (fig. 4.7a and b). CsA also significantly suppressed IL-2 production at all the concentrations tested.

The release of IFNγ and IL-4 was also assessed in T cells treated with the QSSMs and CsA by CBA. 3-oxo-C12-HSL appears to inhibit IFNγ production at 50 μM and 100 μM while CsA appears to suppress IFNγ production in a dose dependent manner with an IC_{50} value of 17 μM. The concentrations of IFNγ obtained for the PQS treated cells exceeded the maximum concentration that could be analysed (> 5000 pg/ml) using the CBA kit. The concentration of IFNγ detected for the anti-CD3/anti-CD28 control also exceeded the maximum concentration therefore statistical analysis could not be calculated for this assay (fig. 4.8).

3-oxo-C12-HSL also significantly inhibited IL-4 release giving an IC_{50} value of 25 ± 5 µM (fig. 4.9) when compared to the anti-CD3 and anti-CD28 control, while PQS inhibited IL-4 production only at 3.12 µM. CsA significantly inhibited IL-4 release at all the concentrations tested (fig. 4.9).
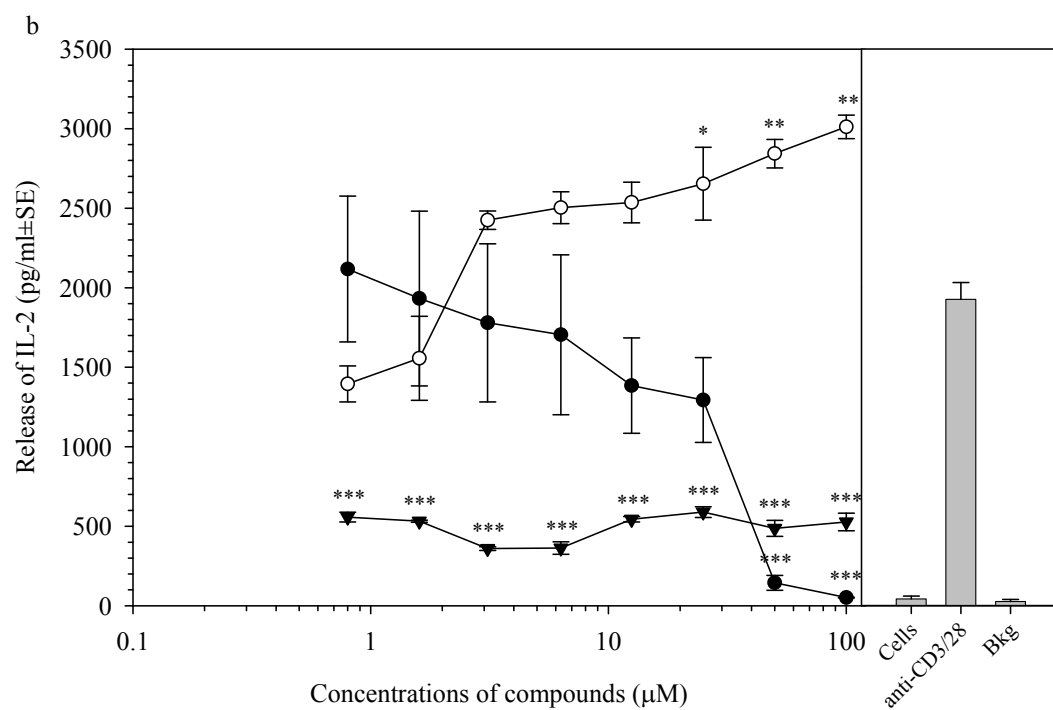
3-oxo-C12-HSL also inhibited the release of IL-5 giving an IC_{50} of 26 ± 1 µM (fig. 4.10) while CsA significantly suppressed IL-5 production in a dose dependent manner giving an IC_{50} value of 1.35 ± 0.05 µM. PQS had no effect on IL-5 production.

In addition, 3-oxo-C12-HSL suppressed IL-10 production with an IC_{50} value of 36 ± 3 µM, whereas PQS significantly inhibited IL-10 release only at 1.6 µM and 3.12 µM. CsA significantly inhibited IL-10 release with near total inhibition at higher concentrations giving an IC_{50} value of 3.5 ± 0.7 µM (fig. 4.11).

Finally, the effect of the QSSMs and CsA on TNFα release was assessed by CBA. 3-oxo-C12-HSL inhibited TNFα production as the concentration increased giving an IC_{50} value of 23 ± 7 µM while PQS significantly enhanced the release of TNFα at concentrations ≥ 25 µM. CsA also significantly inhibited TNFα release with an IC_{50} of 9.7 ± 8.2 µM (fig. 4.12).
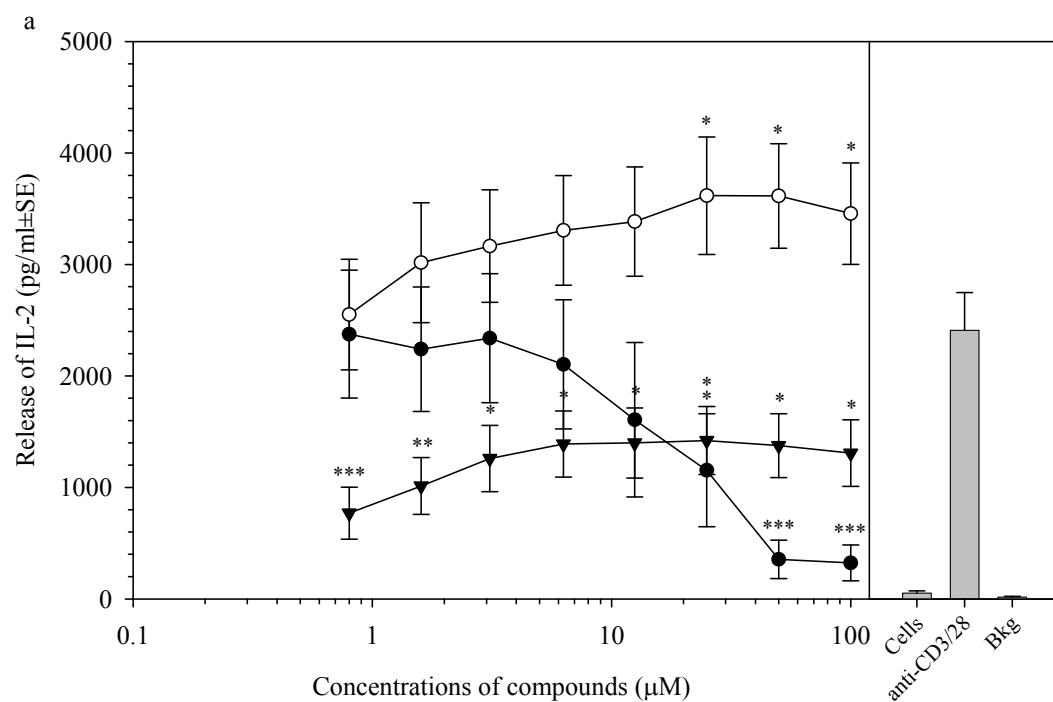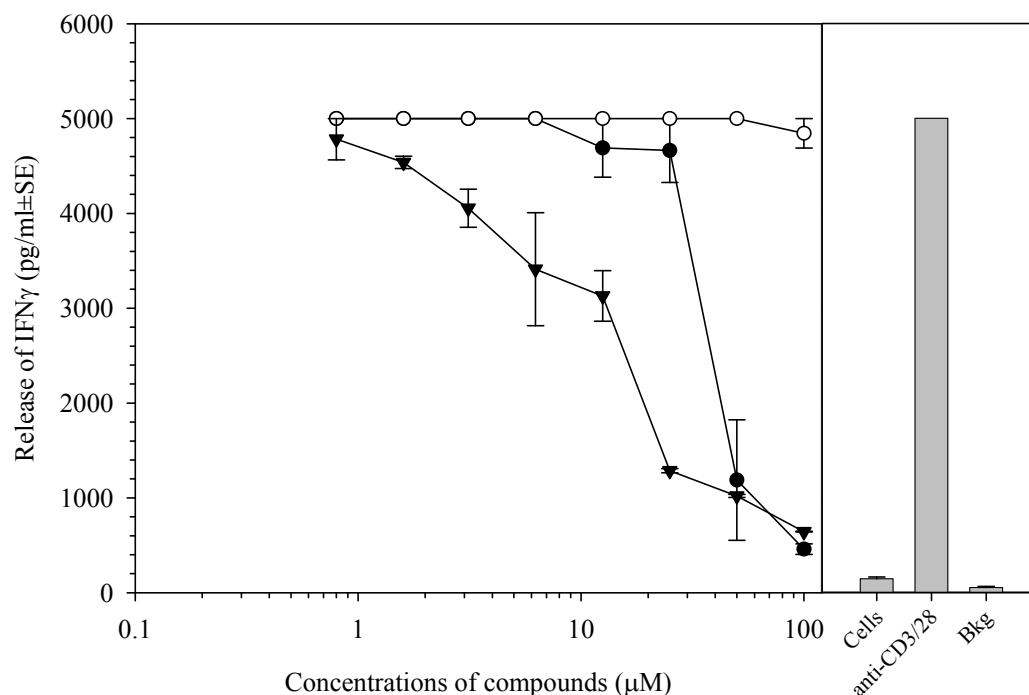
Figure 4.7.

a

Key: 3-oxo-C12-HSL; ● , PQS; ○ , CsA; ▼ .

b

Key: 3-oxo-C12-HSL; ● , PQS; ○ , CsA; ▼ .

Figure 4.7. The effect of *P. aeruginosa* QSSMs and CsA on the release of IL-2 from pure human T cells using the sandwich ELISA technique (a) and CBA technique (b).   3-oxo-C12-HSL inhibited IL-2 release while PQS significantly enhanced the release of IL-2 shown using both the ELISA and CBA techniques. (a) The IC_{50} value obtained for 3-oxo-C12-HSL was 15.7±4.1 µM, while PQS stimulated the release of IL-2 at concentrations above 25 µM. (b) The IC_{50} obtained for 3-oxo-C12-HSL was 28.5±0.5 µM, while PQS stimulated the release of IL-2 at concentrations ≥ 25 µM. Significantly low levels of IL-2 were also found in T cells harvested with cyclosporin A.   ***P<0.001, **P<0.01 and *P<0.05 compared with the anti-CD3/28 control (cells treated with anti-CD3 and anti-CD28 only).   Cells represent untreated cells and CTCM represents background readings.   Results represent the mean of 6 independent experiments for the ELISA assays and 2 independent experiments for the CBA assays. The results of the original experiments are individually plotted in A.2.12 and A.2.13 respectively.
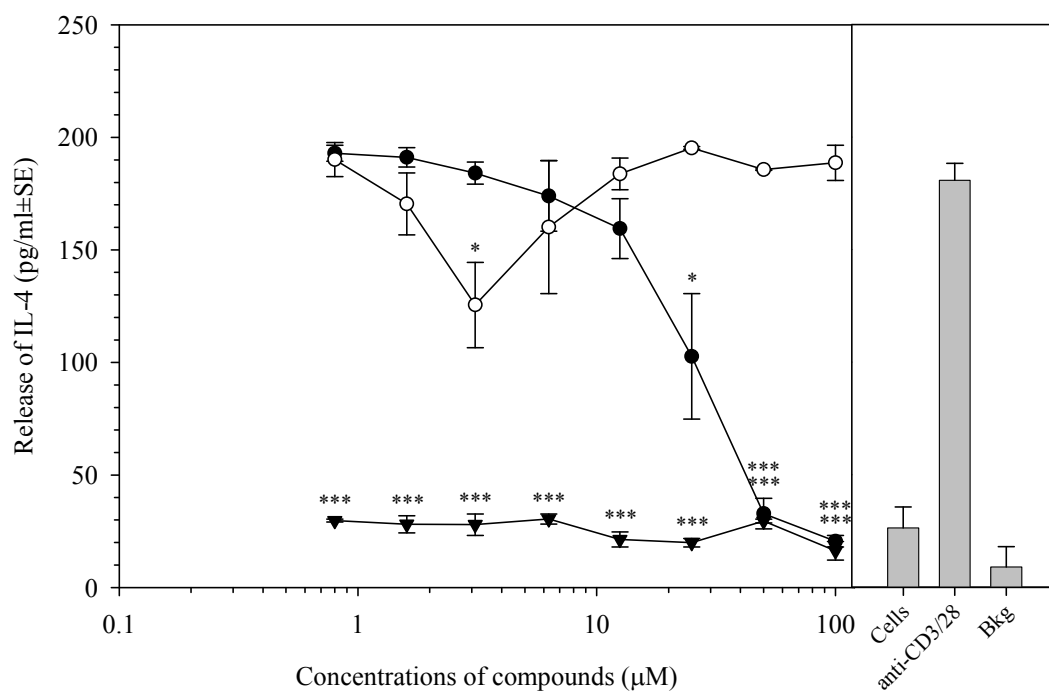
Figure 4.8.

Key: 3-oxo-C12-HSL; ● , PQS; ○ , CsA; ▼ .

Figure 4.8. The effect of *P. aeruginosa* QSSMs and CsA on the release of IFNγ from pure human T cells using CBA technique. The lower concentrations of 3-oxo-C12-HSL (≤ 12 μM) and all concentrations of PQS contained a quantity of IFNγ that exceeded the maximum concentration that could be analysed (> 5000 pg/ml). Higher concentrations of 3-oxo-C12-HSL and CsA appear to inhibit IFNγ release in a dose dependent manner. As the anti-CD3/28 control (cells treated with anti-CD3 and anti-CD28 only) reached over the array limit, it was difficult to apply meaningful statistical analysis to this set of results. Cells represent untreated cells and CTCM represents background readings**.** Data represents the mean of 2 independent experiments (A.2.14).

Figure 4.9.

Key: 3-oxo-C12-HSL; ● , PQS; ○ , CsA; ▼ .

Figure 4.9. The effect of *P. aeruginosa* QSSMs and CsA on the release of IL-4 from pure human T cells using CBA technique. 3-oxo-C12-HSL significantly inhibited IL-4 release with an IC_{50} value of 25±5 µM while PQS had no significant effect on the release of IL-4. CsA significantly inhibited IL-4 release and all concentrations tested. ***P<0.001, *P<0.05 compared with the anti-CD3/28 control (cells treated with anti-CD3 and anti-CD28 only). Cells represent untreated cells and CTCM represents background readings. Data represents the mean of 2 independent experiments (A.2.15).
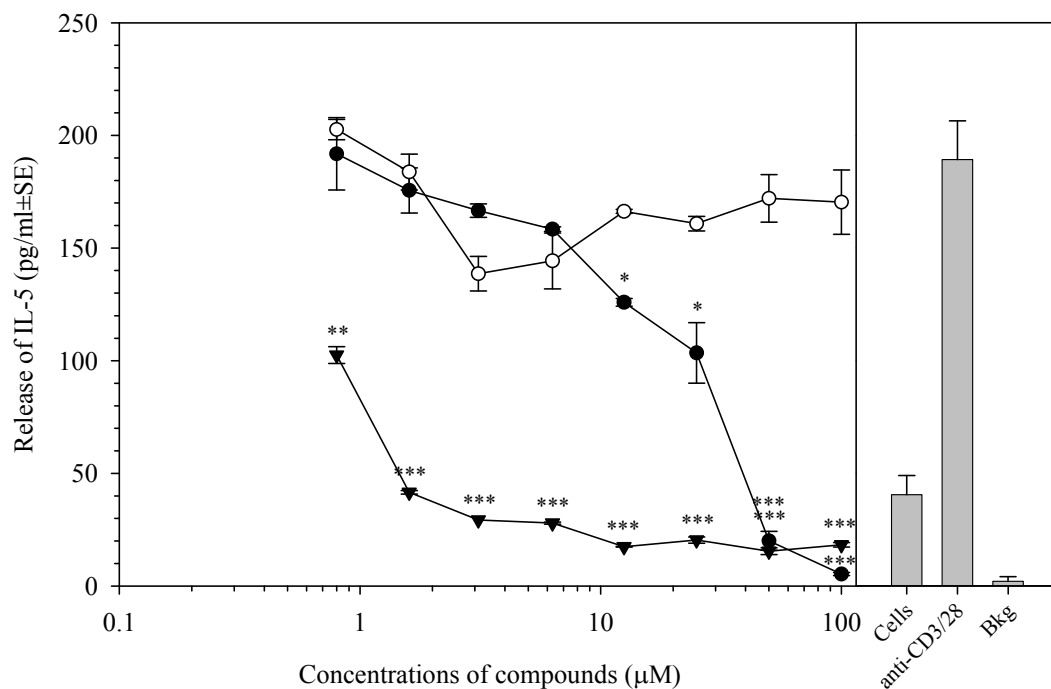
Figure 4.10.

Key: 3-oxo-C12-HSL; ● , PQS; ○ , CsA; ▼ .

Figure 4.10. The effect of *P. aeruginosa* QSSMs and CsA on the release of IL-5 from pure human T cells using CBA technique. 3-oxo-C12-HSL significantly inhibited IL-5 release with an IC_{50} value of 26 ± 1 μM whereas PQS had no significant effect on the release of IL-5. CsA inhibited IL-5 release in a dose dependent manner giving an IC_{50} value of 1.35 ± 0.05 μM. ***P<0.001, **P<0.01 and *P<0.05 compared with the anti-CD3/28 control (cells treated with anti-CD3 and anti-CD28 only). Cells represent untreated cells and CTCM represents background readings. Data represents the mean of 2 independent experiments (A.2.16).
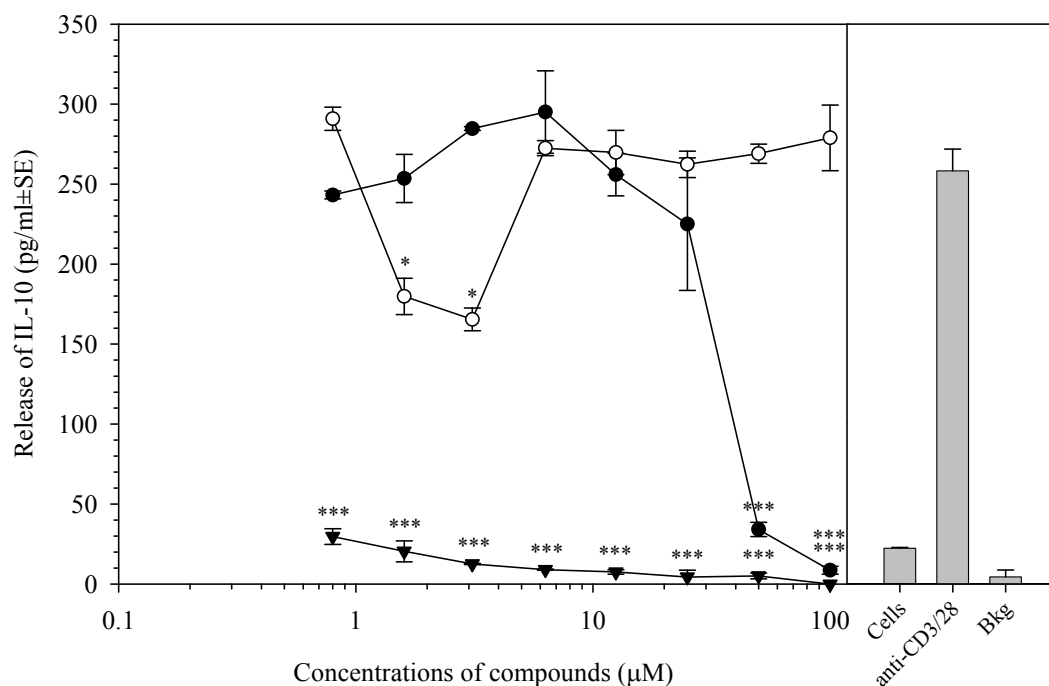
Figure 4.11.

Key: 3-oxo-C12-HSL; ● , PQS; ○ , CsA; ▼ .

Figure 4.11. The effect of *P. aeruginosa* QSSMs CsA on the release of IL-10 from pure human T cells using CBA technique. 3-oxo-C12-HSL significantly inhibited IL-10 release at concentrations with an IC_{50} of 36±3 µM while PQS significantly inhibited IL-10 release only at 3.12 µM. CsA also inhibited the release of IL-10 giving an IC_{50} value of 3.5±0.7 µM. ***$P<0.001$, *$P<0.05$ compared with the anti-CD3/28 control (cells treated with anti-CD3 and anti-CD28 only). Cells represent untreated cells and CTCM represents background readings. Data is represents the mean of 2 independent experiments (A.2.17).
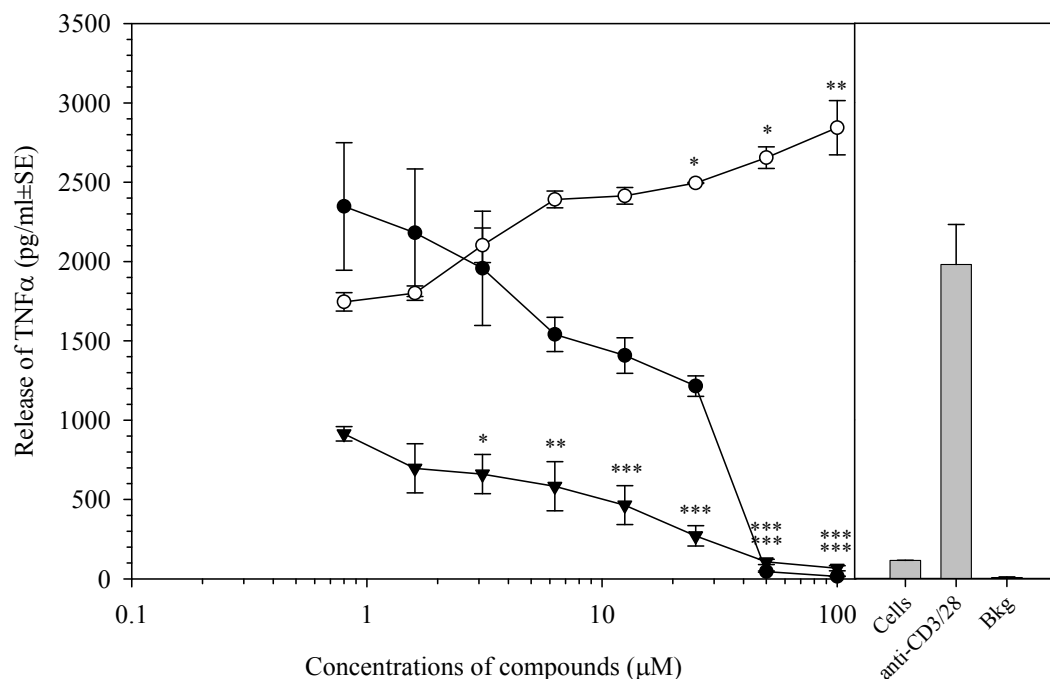
Figure 4.12.

Key: 3-oxo-C12-HSL; ● , PQS; ○ , CsA; ▼ .

Figure 4.12. The effect of *P. aeruginosa* QSSMs and CsA on the release of TNFα from pure human T cells using CBA technique. 3-oxo-C12-HSL significantly inhibited the release of TNFα in a dose dependent manner with an IC_{50} of 23±7 μM whereas PQS enhanced the release of TNFα at concentrations ≥ 25 μM. CsA also inhibited TNFα release giving an IC_{50} value of 9.75±8.2 μM. ***P<0.001, **P<0.01, *P<0.05 compared with the anti-CD3/28 control (cells treated with anti-CD3 and anti-CD28 only). Cells represent untreated cells and CTCM represents background readings. Data represents the mean of 2 independent experiments (A.2.18).

4.3.2.5. Summary of pure T cell assays

Compounds	IC$_{50}$ Anti-CD3 and anti-CD28 proliferation	
3-oxo-C12-HSL	Inhibition	19±1.62 μM
PQS	Inhibition	3.8±0.15 μM
CsA	Inhibition	3.2±0.31 μM
DMSO	No effect	
Ethanol	No effect	

Table 4.2. Summary of the effect of QSSMs and CsA on pure human T cell proliferation.

Cytokines	IC$_{50}$		
	PQS	3-oxo-C12-HSL	CsA
IL-2 (ELISA)	Enhanced release ≥ 25 μM	Inhibition 15.7±4.1 μM	Inhibits at all concentrations
IL-2 (CBA)	Enhanced release ≥ 25 μM	Inhibition 28.5±0.5 μM	Inhibits at all concentrations
IL-4 (CBA)	Inhibition only at 3.12 μM	Inhibition 25±5 μM	Inhibits at all concentrations
IL-5 (CBA)	No effect	Inhibition 26±1 μM	Inhibition 1.35±0.7 μM
IL10 (CBA)	Inhibition only at 1.6 μM & 3.12 μM	Inhibition 36±3 μM	Inhibition IC$_{50}$ = 2.75
IFNγ (CBA)	Inconclusive	Inhibition at high concentrations	Inhibition 17 μM
TNFα (CBA)	Enhanced release ≥ 25 μM	Inhibition 23±7 μM	Inhibition 9.75±8.2 μM

Table 4.3. Summary of the differential effects of 3-oxo-C12-HSL, PQS and CsA on Th1/Th2 cytokine release.

Both 3-oxo-C12-HSL and PQS inhibited pure T cell proliferation with IC_{50} values of 19 ± 1.62 µM and 3.8 ± 0.15 µM respectively. PQS had suppressed T cell proliferation without causing cell death (> 96% cell survival at all PQS concentrations tested) while 3-oxo-C12-HSL inhibited proliferation before cell viability was affected (< 85% cell survival at $\geq$ 25 µM of 3-oxo-C12-HSL). CsA also inhibited T cell proliferation giving an IC_{50} of 3.2 ± 0.31 µM – similar to that of PQS.

3-oxo-C12-HSL inhibited all six cytokines tested where the IC_{50} value obtained for IL-2 is similar to that obtained for the proliferation assay (IC_{50} value of 19 ± 1.62 µM). IL-4, IL-5, IL-10 and IFNγ were also inhibited by 3-oxo-C12-HSL. In contrast, PQS had significantly enhanced IL-2 and TNFα release but inhibited IL-4 and IL-10 at low concentrations, had no effect on IL-5. The positive control, CsA also significantly inhibited all six cytokines where IC_{50} values were obtained for IFNγ, IL-5, IL-10 and TNFα. No IC_{50} values were obtained for IL-2 and IL-4, as the concentrations of these cytokines were similar throughout the CsA concentration range.

4.4. Discussion

The aim of this chapter was to investigate the effects of 3-oxo-C12-HSL and PQS on more biologically relevant cells. The effects of these two QSSMs on human PBMC and pure T cell proliferation, viability and cytokine release was therefore examined. Several reports have focused on the immune modulatory effects of these two QSSMs on mouse splenocytes and human PBMCs but this is the first time their effects have been shown in pure human T cells. As with the mouse splenocyte experiments (chapter 3), CsA was used as the positive control for these human immune cell assays. The concentrations used for the two QSSMs and the incubation time of 48 hr was based on previous studies (Chhabra *et al.*, 2003, Hooi *et al.*, 2004), and allowed both QSSMs to exert their full effects on the cell types used. The concentration and incubation times of CsA were the same as for the QSSMs so that comparable results can be obtained.

4.4.1. Immunosuppressive effects of 3-oxo-C12-HSL and PQS

Human PBMCs and pure T cells were isolated from whole blood donated by healthy individuals. Human PBMCs and pure T cells were stimulated to proliferate and release IL-2 using anti-CD3 and anti-CD28. Engagement of the T cell receptor (TCR)/CD3 complex are involved in signal transduction (Clevers *et al.*, 1988), and anti-CD28 is particularly potent in enhancing lymphokine production (Thompson *et al.*, 1989). The CD28 receptor is expressed on the surface of most mature T cells; it is found on approximately 95% of CD4$^+$ and on 50% CD8$^+$ T cells (Damle *et al.*, 1983). Studies have shown that co-stimulation of CD3 with CD28 leads to complete

activation of T cells and a dramatic up-regulation of IL-2 expression mediated by both enhanced transcription and induction of mRNA stabilisation (Fraser *et al.*, 1991, Lindsten *et al.*, 1989), confirming the requirement of anti-CD3 and anti-CD28 monoclonal antibodies for T cell activation in this present research.

In the human immune system, activation of the T lymphocyte upon recognition of foreign antigen and presentation of the foreign fragment and MHC class to the TCR via APCs, another protein is brought into the receptor complex: the CD4 or CD8 molecule depending on the type of foreign fragment. CD4 binds MHC class II molecules and therefore participates in the response to antigen by inducer T cells, whereas CD8 binds MHC class I molecules, and therefore participates in the response to antigen by cytotoxic T cells (Swain, 1983). These 'accessory' molecules are not always essential for antigen responsiveness as past studies have shown activation of T cells occurs in the absent of CD4 or CD8 binding (Macdonald *et al.*, 1982). Instead, they are thought to facilitate the response of T cells bearing receptors with low affinity for the peptide-MHC complex by increasing the avidity of the T cell for the APC, by altering the signalling portion of the antigen receptor to decrease the occupancy required for signal transduction, or by transmitting a separate synergising signal (Eichmann *et al.*, 1987), therefore the addition of these molecules were not required for T cells activation in this study.

Both 3-oxo-C12-HSL and PQS, as well as the positive control CsA, suppressed human PBMC and pure T cells proliferation (fig. 4.2 and fig. 4.6a respectively). PQS was the more potent anti-proliferative compared to 3-oxo-C12-HSL as indicated by the IC_{50} values obtained, 3.4 ± 0.34 µM and 42.4 ± 4 µM respectively for the human

PBMC assay and 3.8±0.15 µM and 19±1.62 µM respectively for the pure T cell assays. This supports previous studies demonstrating the immune-suppressive properties of these two QSSMs on human PBMCs (Hooi *et al.*, 2004), and this is the first time their immune-suppressive properties have been shown in pure human T cells. The immunosuppressive drug CsA gave IC_{50} values similar to PQS – 5.1±0.35 µM in the human PBMC assays and 3.2±0.31 µM in the pure T cell assay. Therefore, even though 3-oxo-C12-HSL appears to exhibit a similar mode of action to CsA (Hooi *et al.*, 2004), PQS seems to have a similar potency to CsA.

Previous studies have looked at the effects of CsA on human T cell proliferation. Umland *et al.* noticed that stimulation in the presence or absence of anti-CD28 resulted in distinctly different responses to CsA. Almost complete inhibition of proliferation was seen with anti-CD3-stimulated T cells in the presence of > 100 ng/ml CsA with an IC_{50} of 10 ng/ml. In contrast, when T cells were stimulated with both anti-CD3 and anti-CD28, there was at least a 10 fold decrease in potency of CsA compared with that of T cells stimulated with anti-CD3 alone (Umland *et al.*, 1999). Additionally, Nishiyama S *et al.* reported 10 ng/ml of CsA reduced T cell proliferation by 50% compared to anti-CD3 and anti-CD28 stimulated T cells without the addition of CsA (Nishiyama *et al.*, 2005). The decrease in CsA potency to anti-CD3 and anti-CD28 stimulated cell proliferation may be due to proliferation induced by the CD28 pathway is resistant to CsA (June *et al.*, 1987).

The differences in IC_{50} values obtained between human PBMCs and pure T cells assays, especially seen with 3-oxo-C12-HSL, may reflect the ability of the QSSMs to concentrate on targeting the signalling pathways involved in T cell proliferation and

the ability to calculate a more precise value because of the purity of the T cells isolated (93%, fig. 4.5).

MTS assay data illustrates 3-oxo-C12-HSL suppressed cell proliferation before cell viability was affected, whereas PQS and CsA inhibited human PBMC and pure T cell proliferation in the absence of cytotoxicity (fig. 4.3 and 4.6b respectively). The reduction of cell proliferation was therefore due to an inhibitory effect by the QSSMs and not cell death. In addition, an increase in absorbance was observed when cells were treated with high concentrations of PQS. Since PQS was shown to inhibit T cell proliferation, the increase in absorbance was therefore not due to an increase in viable cell concentration. There are several explanations for this. PQS at high concentrations may cause the increase in absorbance itself, therefore, for future reference, extra controls should be included in this study, for example, testing the absorbance of medium with the different concentrations of PQS to compare with the absorbance of cells treated with PQS. In addition, PQS may have caused in increase in metabolism in the cells. The MTS/PMS assay relies on the CellTiter colourless reagent being reduced by viable cells into a coloured compound. However, if PQS caused an increase in cellular metabolism, more of the CellTiter reagent being reduced by cells can be seen without an actual increase in the number of viable cells.

4.4.2. Effects of 3-oxo-C12-HSL and PQS on T cell cytokine release

To gain further insight into the molecular mechanisms of action of 3-oxo-C12-HSL and PQS, the effects of these QSSMs on cytokine production in T cell proliferation was investigated.

As one of the hallmarks of T cells activation by engagement of TCR/CD3 complex and CD28 is the production and release of IL-2, the levels of IL-2 released from anti-CD3 and anti-CD28-stimulated human PBMC and pure T cells in the presence of the QSSMs and CsA were tested to further confirm the activation of T cells by anti-CD3 and anti-CD28.

Activated T cells induce mRNA for IL-2 and produce IL-2 protein; secreted IL-2 successfully induces T cell proliferation by binding to the IL-2 receptor present on activated T cells (Geppert and Lipsky, 1987, Meuer *et al.*, 1984). Results indicate PQS, in contrast to 3-oxo-C12-HSL, did not inhibit the release of IL-2 in both human PBMC and pure human T cells (fig. 4.4 and 4.7 respectively). Indeed, PQS induced a small but significant increase in the release of IL-2 above the anti-CD3 and anti-CD28 control stimulations while still inhibiting cell proliferation in the pure human T cell assay, suggesting a cytostatic effect and supporting previous suggestion that PQS active proximately to the IL-2R or downstream of the IL-2R in the T cell signalling pathway. 3-oxo-C12-HSL inhibited the release of IL-2 giving an IC_{50} value of 18±4 μM in the human PBMC assays and 15.7±4.1 μM and 28.5±0.5 μM in the pure T cell assays using ELISA and CBA analysis respectively, supporting the finding that 3-oxo-C12-HSL acts on early T cell events (Ritchie *et al.*, 2005). A significant enhancement of IL-2 was not seen in the human PBMC compilation graph (fig. 4.4) which may seem to contradict findings by Hooi *et al.*. However, a significant increase in IL-2 can be seen in the individual data (A.2.9).

CsA also suppressed IL-2 release in both human PBMC and pure T cell assays. Past studies have demonstrated the inhibitory effect of CsA on IL-2 production. Landewé

et al. showed that 1-100 nM inhibited the production of IL-2 in a concentration-dependent fashion with an IC_{50} of approximately 11 nM. In addition, cloned T cell activated with anti-CD3 in the presence o IL-2 and CsA resulted in a concentration restoration of proliferative activity to the level of drug-free anti-CD3 induced proliferative activity (Landewe *et al.*, 1995). The reversibility of the CsA-induced inhibition of T cell proliferation by IL-2 suggests that inhibition of IL-2 production by CsA is responsible for the effect of CsA on T cell proliferation.

Using CBA analysis, 3-oxo-C12-HSL seems to inhibit production of IFNγ in anti-CD3 and anti-CD28 stimulated pure T cells, a cytokine required for the promotion of Th1 linage (fig. 4.8), though results were inconclusive for PQS due to limitations using the CBA kit. Nevertheless, inhibition of IFNγ by 3-oxo-C12-HSL does confirm data obtained from mouse splenocyte experiments. In addition, 3-oxo-C12-HSL also suppressed IL-4 release, a cytokine required to stimulate a Th2 response, while PQS only inhibited IL-4 production at 3.12 μM (fig. 4.9). The inhibition of IFNγ and IL-4 by 3-oxo-C12-HSL may indicate it is a less specific inhibitor of T cells function due to the suppression of both Th1 and Th2 linage, compared to PQS which only inhibited IL-4 production at a low concentration and was shown to inhibit IFNγ in mouse splenocytes, suggesting it may suppress T cell activation primarily via the Th1 signalling pathway. CsA also inhibited IFNγ with an IC_{50} value of 17 μM and inhibited IL-4 at the concentrations tested. Past studies have also demonstrated the inhibition of IL-4 and IFNγ. Umland *et al.* showed inhibition of IFNγ and IL-4 by CsA with IC_{50} concentrations of 40 to 60 ng/ml (Umland *et al.*, 1999), however, the concentration of CsA used in their study was considerably lower (1-100 ng/ml) than those used in this present study.

It was further demonstrated 3-oxo-C12-HSL and CsA suppressed IL-5 production with IC_{50} value of 26 ± 1 μM and 1.35 ± 0.05 μM respectively, while PQS had no effect on this cytokine (fig. 4.10). Inhibition of IL-5, a Th2 cytokine, prevents interaction with T lymphocytes to mediate maturation of cytotoxic T cells and further indicates the suppression of a Th2 response. Umland *et al.* showed that CsA-mediated inhibition of IL-5 production by $CD4^+$ T cells stimulated with anti-CD3 and anti-CD28 was biphasic. CsA concentrations of less than 125 ng/ml, IL-5 levels were enhanced above that found in the control cultures. Concentrations of CsA above 250 ng/ml were weakly inhibitory (Umland *et al.*, 1999). The concentrations used in this present study were considerably higher ($\geq$ 0.8 μM) and were shown to be strongly inhibitory.

In addition, 3-oxo-C12-HSL and PQS both inhibited the release of IL-10 though PQS only significantly inhibited this cytokine at 1.6 μM and 3.12 μM (fig. 4.11). IL-10 is produced in both recently differentiated primary Th1 and Th2 cells, although Th2 cell produce much higher levels than Th1 cells. As naïve T cells differentiate to Th1 and Th2 cells, Th1 cells slowly lose their ability to express IL-10, whereas Th2 cells increase IL-10 production and maintain high IL-10 levels. Furthermore, IL-10 inhibits Th1 proliferation by suppressing IFNγ and IL-2 release. IL-10 also stimulates and/or enhances the proliferation of B cells, thymocytes and mast cells (Moore *et al.*, 1993) and enhances the cytotoxic T cell generation (Yang *et al.*, 1995a). Inhibition of IL-10 therefore indicates a preference to allow Th1 proliferation to occur, again suggesting suppression of a Th2 response by 3-oxo-C12-HSL and to some extent, by PQS. CsA also suppressed IL-10 release with an IC_{50} of 3.5 ± 0.7 μM. Rafiq *et al.* recognized that IL-10 production was

strongly, although not completely, blocked by CsA at 400 ng/ml in T cells stimulated with anti-CD3 cross-linked on P815/CD80 cells (Rafiq *et al.*, 1998) and is in agreement with other published data showing that the immunosuppressive drug CsA inhibits IL-10 production in murine T cells *in vitro* (Tomkins *et al.*, 1995) and *in vivo* (Durez *et al.*, 1993). Moreover, circulating levels of IL-10 were suppressed by treatment with CsA in patients with rheumatoid arthritis (Ferraccioli *et al.*, 1998).

Further differential immune modulatory activity was observed when TNFα secretion was inhibited by 3-oxo-C12-HSL and CsA yet PQS had a stimulatory effect (fig. 4.12). Previous studies have demonstrated intracheal infection of mice with *P. aeruginosa* leads to up-regulation of TNFα mRNA expression and enhanced secretion of TNFα protein into the alveolar space, and were associated with host resistance to infection, suggesting that TNFα may represent an important genetically regulated early defence signal crucial for the outcome of acute infection with *P. aeruginosa*. This was further supported by the finding that *in vivo* depletion of TNFα markedly impaired the ability of BALB/c mice to control infection with *P. aeruginosa* (Gosselin *et al.*, 1995). TNFα is also known to promote the recruitment of neutrophils and macrophages to the site of infection (Kolls *et al.*, 1993, Vanfurth *et al.*, 1994). This effect was shown to be associated with the capacity of TNFα to up-regulate the expression of adhesion molecules on endothelial and epithelial cells as well as neutrophils (Mulligan *et al.*, 1993, Tosi *et al.*, 1992), to stimulate the secretion of chemotactic factors by epithelial cells and phagocytes (Smart and Casale, 1994), or to act directly as a chemoattractant (Ming *et al.*, 1987). The ability to inhibit TNFα is therefore advantageous to the bacterium. However, a number of reports have also demonstrated that high levels of TNFα sustained over a

prolonged period may cause severe lung damage, shock, fibrosis and tissue dysfunction (Remick, 1993, Kolls *et al.*, 1993, Piguet *et al.*, 1990, Tracey and Cerami, 1994).

It is generally accepted that cytokines produced by Th1 cells, particularly IFNγ, activate phagocytic cells. Th1 cell when activated produce chemokines that assist in the recruitment of monocytes, and GM-CSF that induces their differentiation into macrophages at the site of infection. TNFα from Th1 cells alters the surface properties of endothelial cells to promote the adhesion of monocytes at the site of infection. IFNγ and TNFα are then critical for effective macrophage activation and the elimination of the pathogen. In addition, Th1 cytokines especially, IFNγ and TNFα, help the development of B cells and the production of IgG1 and IgG3 opsonising - which provide the bulk of immunity to most blood borne infectious agents - and complement-fixing antibodies (Abbas *et al.*, 1996, Romagnani, 1994). In contrast, cytokines produced by Th2 cells provide optimal help for humoral immune responses. Th2 cytokines, especially Il-4 and IL-5 induce IgE production by B cells through induction of mast cells and eosinophil differentiation and activation and facilitation to IgA synthesis (Abbas *et al.*, 1996, Romagnani, 1994). Allergic reactions are predominantly associated with IgE, where reintroduction of a antigen into a previously sensitised individual binds to antigen-specific IgE on mast cells and trigger the release of pharmacologically active agents e.g. histamine (Alberts *et al.*, 1994, Janeway *et al.*, 2001). On the other hand, IgA is a first line of defence against microbes entering through mucosal surfaces (the respiratory, gastrointestinal and genitourinary tracts). This antibody prevents colonisation of mucosal surfaces by pathogens and mediates their phagocytosis (Alberts *et al.*, 1994).

The cytokine results obtained therefore indicate elimination of both Th1 and Th2 responses by 3-oxo-C12-HSL and PQS. The ability to inhibit one or both pathways will therefore help promote and sustain bacterial infections.

To conclude, 3-oxo-C12-HSL and PQS are immunosuppressive in that they suppress proliferation of T cells and modulate cytokine release, where PQS is significantly more potent. However, while PQS has no cytotoxic effects on T cells, 3-oxo-C12-HSL inhibited proliferation before cytotoxicity occurred. These two structurally diverse QSSMs can also exert differential modulatory effects on pure human T cell response, most noticeably on the cytokines IL-2 and TNFα, where 3-oxo-C12-HSL inhibits and PQS stimulates IL-2 and TNFα production. As T cell play an important role in defending the host against *P. aeruginosa* infections, it is important to understand in more detail the mode of action of these two QSSMs at a protein and RNA level.

5.   The effect of *P. aeruginosa* PQS on the activation of Erk 1/2

5.1. Introduction

Hooi *et al.* (2004) has suggested 3-oxo-C12-HSL and PQS possess activities reminiscent of CsA and rapamycin on hPBMCs. It has been well established that rapamycin prevents T cell proliferation by blocking IL-2 signalling (Powell *et al.*, 1998, Brennan *et al.*, 1999). Rapamycin inhibits the serine-threonine kinase mTOR (Brown *et al.*, 1994, Almawi *et al.*, 1999, Gingras *et al.*, 2001) where mTOR is a key regulator of translation. CsA, on the other hand inhibits phosphatase activity of calcineurin (Liu *et al.*, 1991) thus inhibiting the translocation of the NFAT family of transcription factors important for IL-2 production. CsA has also recently been found to block JNK and p38 signalling pathways (Matsuda and Koyasu, 2000). The potential for 3-oxo-C12-HSL and PQS to become future immunosuppressants due to their immune suppressive properties warrants further research into their mode of action in the T cell signalling pathway. In addition, the importance of MAPKs in controlling cellular responses to the environment and in regulating gene expression, cell growth and apoptosis has made them a priority for research related to many human diseases (Kawaguchi *et al.*, 2003, Zhang *et al.*, 2005, Zhu *et al.*, 1999, Zhang *et al.*, 1999).

The Erk pathway is a molecular target for drug development supporting the reason to test the effects of the QSSMs on this particular signalling pathway. Smith *et al.* (2001) demonstrated 3-oxo-C12-HSL activated Erks that subsequently induced the activation of transcription factor NFκB in bronchial epithelial cells. The Erk

signalling pathway is also an important element in Nerve growth factor (NGF) survival. NGF is a well-characterised neurotrophin essential for development and differentiation of both peripheral and central neurones. NGF also exerts specific effects on immune functions and previous studies have given evidence that NGF promotes T lymphocyte proliferation (Thorpe and Perezpolo, 1987, Otten *et al.*, 1989). The binding of NGF to the TrK receptor tyrosine kinase A (TrKA) causes its dimerisation leading to the phosphorylation of the TrKA receptor. TrK phosphorylation induces the activation of three major intracellular signalling pathways; the PI3-kinase pathway (resulting in Akt phosphorylation), the Ras pathway (resulting in Erk 1/2 phosphorylation), and the phospholipase Cγ pathway (Kaplan and Miller, 2000) (fig. 5.1). Ehrhard *et al.* (1994) demonstrated expression of functional NGF receptors on T cells (Ehrhard *et al.*, 1994), and is consistent with data showing that NGF induces a proliferative response in human blood peripheral T lymphocytes (Otten *et al.*, 1989) and in rat spleen lymphocytes (Thorpe and Perezpolo, 1987), and stimulates expression of IL-2R on cultured human lymphocytes (Thorpe *et al.*, 1987). Taken together, suggest that NGF, in addition to its neurotrophic functions, acts as an immunomodulator mediating cross-talk between cells of the nervous and the immune systems. The inhibition of the NGF pathway will therefore also be advantageous to *P. aeruginosa*.

Using this as a starting point, the effect of 3-oxo-C12-HSL and PQS on total and phospho-Erk 1/2 in pure T cells were investigated using western blot analysis. SDS-PAGE separates proteins based primarily on their molecular masses (Laemmli, 1970). SDS is an anionic detergent, meaning that when dissolved its molecules have a net negative charge within a wide pH range. A polypeptide chain binds SDS in

proportion to its relative molecular mass. The negative charges on SDS denature most of the complex structure of proteins, which are strongly attracted toward an anode (positively-charged electrode) in an electric field. Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample.
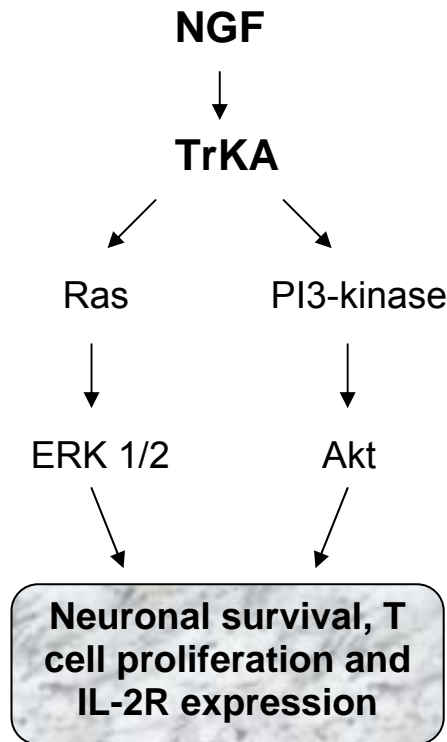
Figure 5.1

NGF

↓

TrKA

Ras   PI3-kinase

ERK 1/2   Akt

Neuronal survival, T cell proliferation and IL-2R expression

Figure 5.1. NGF signalling pathway. NGF is the ligand of TrKA and induces its phosphorylation and the subsequent activation of the intracellular signalling pathways Erk 1/2 and Akt, which are responsible for neuronal survival as well as T cell proliferation (Akassoglou, 2005).

5.2. Methods

5.2.1. Immunoblotting

T cells were isolated and cell density determined using the trypan blue assay. 500 μl of cell suspension was added at a density of 10^6 cells per well of 48-well tissue culture plates (NUNC, Leicestershire, UK). 4 μM PQS was added per well after the addition of 0.1 μg/ml anti-CD3 and 5 μg/ml anti-CD28 bringing the final volume up to 1ml per well with CTCM. T cells were incubated for 0, 5, 10, 20, 30, 60, 90 and 120 min. Resting control (T cells with no stimulants) was also included in this assay. Cells were then collected into eppendorfs and centrifuged for 10 min at 450 x g, discarding the supernatant and washing three times with cold phosphate buffered saline (PBS, A.3.1), centrifuging at 10 000 x g for 5 min each time. After removing the final supernatant, cells were lysed with RIPA buffer (Sigma, Poole, UK) in the presence of protease inhibitors (Sigma, Poole, UK) and 1 μl of phosphatase inhibitor I and II (Sigma, Poole, UK) for 30 min on ice. Cells were then centrifuged at 12 000 x g for 30 min at 4°C and the supernatant containing the total cellular extracts were collected. Protein concentration was measured using the NanoDrop Spectrophotometer (Labtech International, Sussex, UK) at the wavelength OD_{280}. The cellular extracts were collected using acetone precipitation. Briefly, ice cold acetone (Fisher Scientific, Leistershire, UK) was added to the total cellular extracts in RIPA buffer at the ratio of 4:1 respectively and incubated overnight at -20°C, followed by centrifugation at full speed 10 000 x g for 15 min, discarding the supernatant and re-suspending the pellet in SDS reducing sample buffer (1 M Tris-HCL pH 6.8, 50% glycerol, 10% SDS, 1% bromophenol blue and 0.5 ml 2-ME)

at a volume used to dilute the protein concentration to 50 μg per 20 μl. Proteins were then denatured by boiling the sample at 100°C for 10 min and electrophoresed on SDS-PAGE gel.

12% polyacrylamide (Sigma, Poole, UK) gel was used as resolving gel (29.2% acrylamide, 1.5 M Tris-HCl pH 8.8, 10% SDS, 60 μl 10% ammonium persulphate and 6 μl TEMED) and 4% was used as stacking gel (29.9% acrylamide, 0.5 M Tris-HCL pH 6.5, 10% SDS, 50 μl 10% ammonium persulphate and 20 μl TEMED). The Bio-Rad Mini-Gel apparatus was setup by clamping clean back and front plates together and placing in casting stand. After checking if there was any leakage with water, the resolving gel was prepared and poured in between the two plates overlaying with tert-amyl alcohol and allowing it to polymerize for 30 min. Residual alcohol and buffer mix was poured off and the surface of the gel washed with dH$_2$O several times draining dH$_2$O completely. The stacking gel was now prepared and 900 μl was poured above the resolving gel before introducing a clean dry comb at an angle to avoid trapping bubbles and left to polymerize for 10 min. The comb was removed from the set gel transferring the gel to an electrode stand and placed in a tank, pouring Running Buffer (A.1.5.4) into the tank washing out the well with reservoir buffer using a pipette. 20 μl of protein sample was loaded into each well including a well with 10 μl of pre-stained standards with a weight range of 10-250 kDa (Bio-Rad, Hertfordshire, UK). The red and black electrical leads in the green cover was connected to the matching coloured electrodes of the electrode assembly and ran at 100 V for 90 min until the bromophenol blue dye has migrated to the bottom of the gel.

When finished, the proteins were transferred from SDS-PAGE gel onto nitrocellulose membrane (Towbin *et al.*, 1979) using a Bio-Rad Mini Protean II/III Transfer cell. Briefly, a sandwich composed of filter paper-gel-blot (nitrocellulose)-filter paper was made and placed in a tank with transfer buffer (A.1.5.5) giving an electric field where negatively charged proteins migrate out of gel onto the nitrocellulose blot. Transfer was carried out at 30 V overnight. The blot was removed from the transfer apparatus, rinsed with Tris buffered saline (A.1.6.1), and immediately placed into blocking buffer (TBS buffer/1% BSA) for 2 hr at 37°C then washed three times with 75 ml TBS supplemented with 0.05% Tween-20 (TBS/T, Sigma, Poole, UK) for 15 min each with agitation. Primary antibody (Promega, Southampton, UK) was diluted to a ratio of 1:5000 with TBST/0.1% BSA and incubated with the membrane at 4°C overnight with agitation. The blot was then washed three times with 75 ml TBS/T for 15 min each with agitation before incubating with donkey anti-rabbit IgG AP-conjugate (Promega, Southampton, UK) diluted to a ratio of 1:5000 with TBST/0.1% BSA for 2 hr at room temperature with agitation. The membrane was washed three times (15 min each wash in 75 ml TBS/T buffer) and rinsed twice for 1 min each in TBS buffer decanting the solution after each wash. Chemiluminescent detection was used to analyse the gel results on nitrocellulose membrane. Alkaline phosphatase substrate (50 mg/ml 5-bromo-4-chloro-3-indoly phosphate in 70% PMF, 70 mg/ml nitro blue tetrazolium in 70% PMF and 0.75 M Tris pH 9.6) was used as detection, removing excess reagent with distilled water before scanning the blot.

The blotted membranes were scanned using the Bio-imaging system (Syngene, Cambridge, UK). Activation of Erk 1/2 was determined by normalization of band density from the phosphorylated protein with that of the total (phosphorylated and

non-phosphorylated) protein for the same sample using GeneTools Image Analysis software (Syngene, Cambridge, UK).

5.3. Results

The presence of total-Erk 1/2 was detected when cells were treated with 4 µM PQS, however, there was no indication of PQS having any effect on the concentration of total-Erk 1/2 present (fig. 5.2a and b). It had been previously suggested that *P. aeruginosa* QSSMs may interfere with the activation of Erk 1/2 and so the effect of 4 µM of PQS on phospho-Erk 1/2 was investigated. Figure 5.3a and b indicates the presence of phospho-Erk 1/2 however, no significant changes to their concentration was detected within the time scale the T cells were treated.
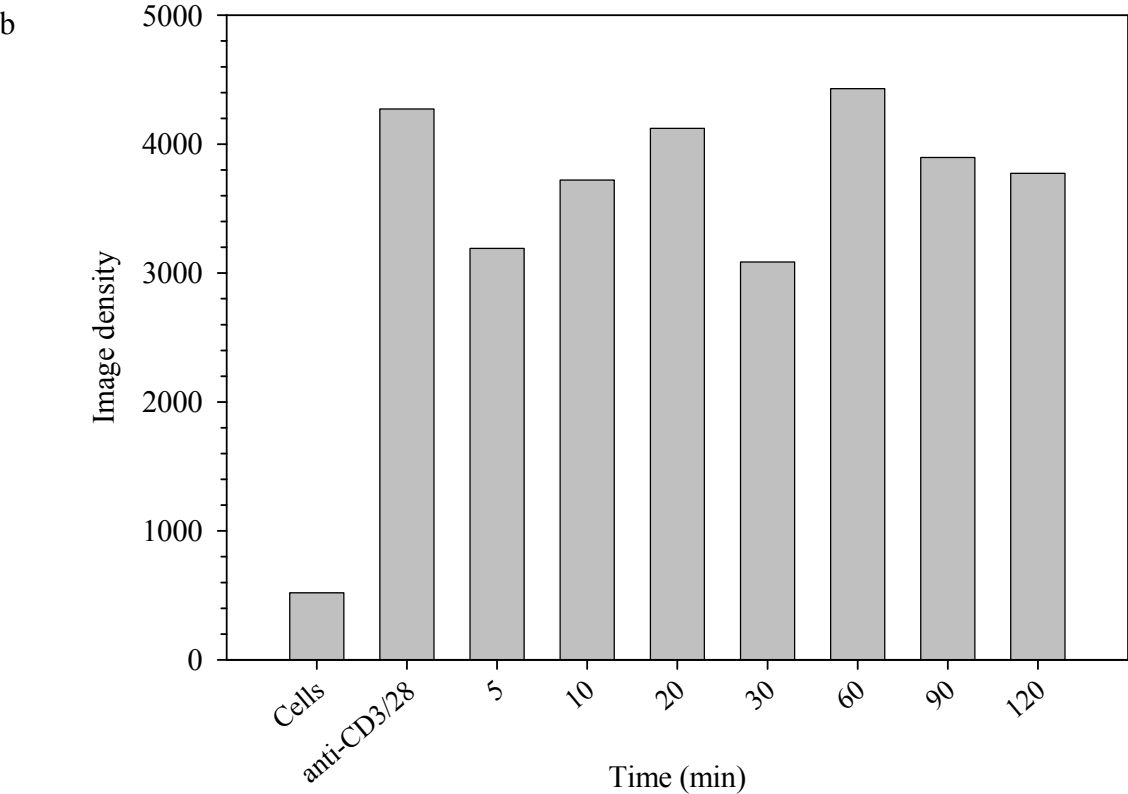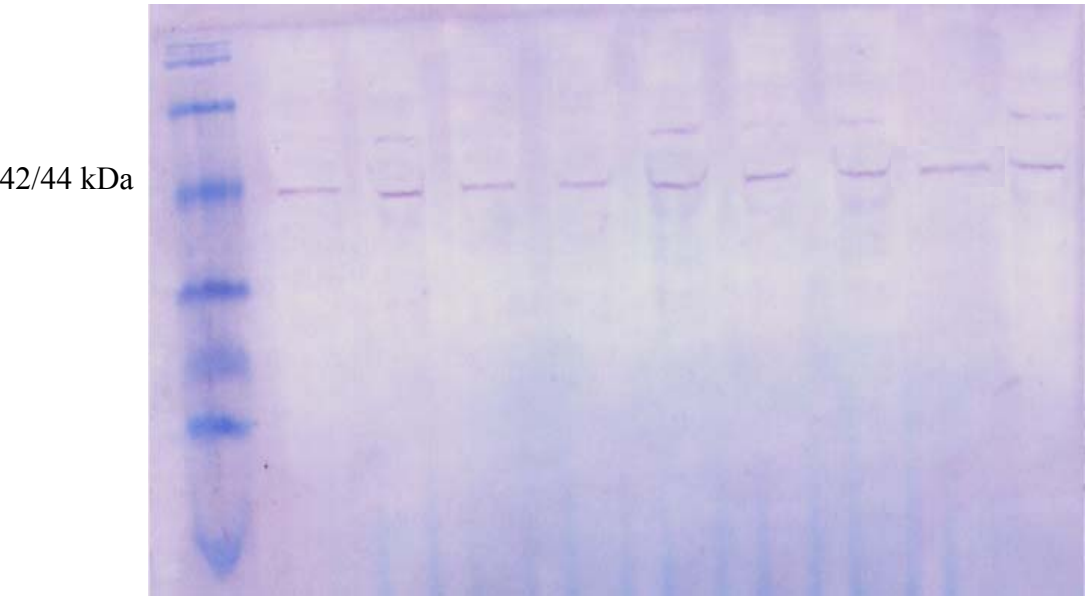
Figure 5.2

a

b
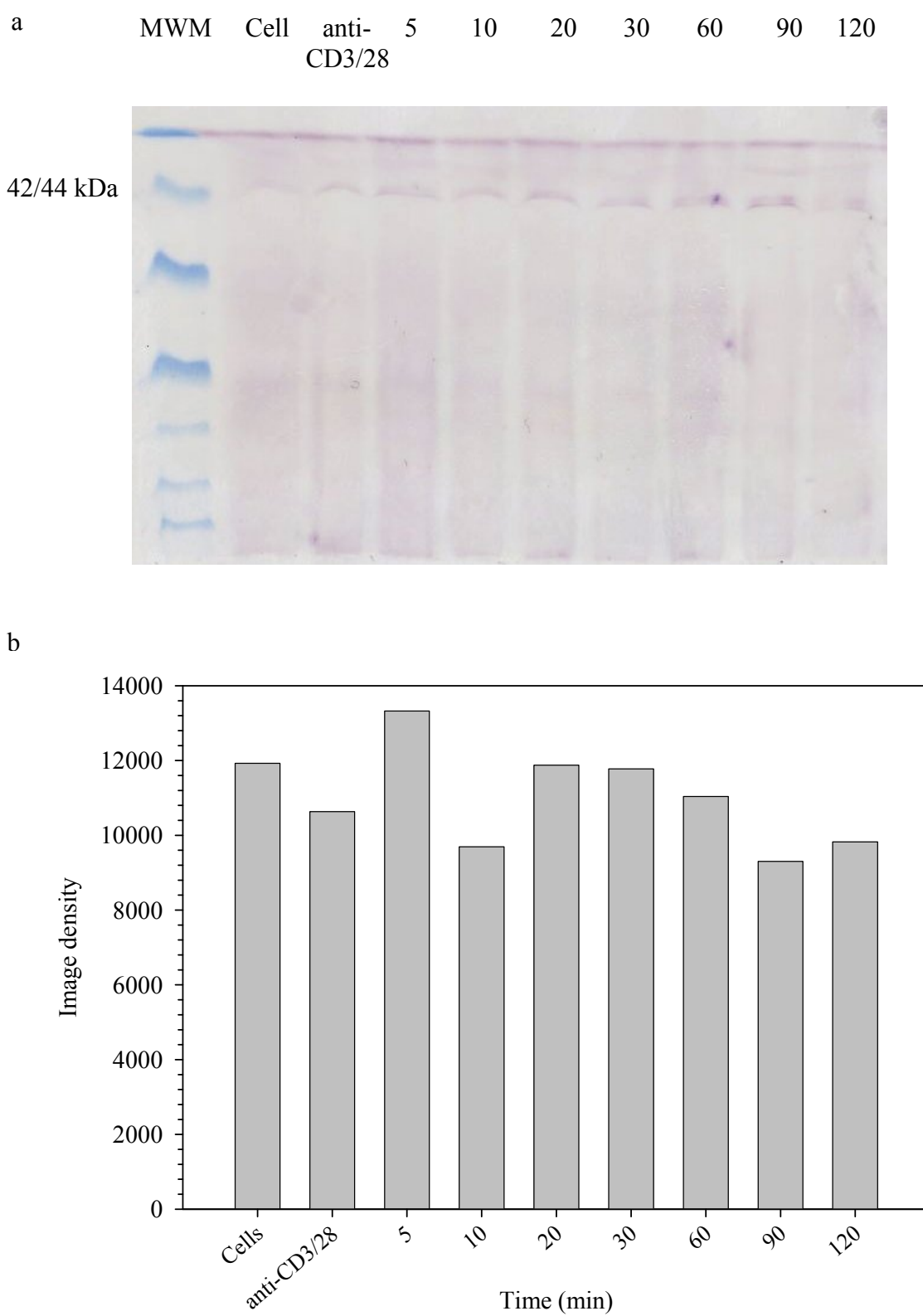
Figure 5.2. Detection of inactivated (total)-Erk 1/2 in pure human T cells stimulated anti-CD3 and anti-CD28. Cells were treated with 4 μM PQS and incubated for 5-120 min. Cells represent untreated cells and anti-CD3/28 represents cells treated with anti-CD3 and anti-CD28 only – both incubated for 120 min. Bands were compared to pre-stained protein ladder (MWM) to confirm the location of total-Erk (a). It was confirmed there were no significant changes in total-Erk quantity compared to the stimulated control using GeneTools image analysis software (b).

Figure 5.3

a.

b

Figure 5.3. Detection of activated (phospho)-Erk 1/2 in pure human T cells stimulated with anti-CD3 and anti-CD28. Cells were treated with 4 μM PQS and incubated for 5-120 min. Cells represent untreated cells and anti-CD3/28 represents cells treated with anti-CD3 and anti-CD28 only – both incubated for 120 min. Bands were compared to pre-stained protein ladder (MWM) to confirm the location of total-Erk (a). It was confirmed there were no significant changes in phospho-Erk present compared to the stimulated control using GeneTools image analysis software (b).

5.4. Discussion

Figure 4.2b shows a constant density of total Erk 1/2 is present throughout T cells stimulated to proliferate with anti-CD3 and anti-CD28 and further treated with 4 μM PQS. Figure 4.3 indicates the presence of phospho-Erk 1/2 in pure human T cells stimulated to proliferate using anti-CD3 and anti-CD28 and again treated with 4 μM PQS. However, it was expected a decrease in phospho-Erk 1/2 to coincide with the inhibition of T cell proliferation. Reasons why this did not occur may be that the activation and progression of the T cell signalling pathway is so fast when the T cells were treated with PQS after the addition of anti-CD3 and anti-CD28, the signalling pathway may already have occurred. Previous studies have shown the presence of phosphor-Erk 1/2 in anti-CD3 and anti-CD28 stimulated human T cells within 1 min from stimulation, with strong activation within 10 min and a decreased presence of phospho-Erk 1/2 within 30 min using western blot analysis (Zaffran *et al.*, 2001). Therefore, future research in repeating this study may need to add the PQS in first before the addition of anti-CD3 and anti-CD28 to activate T cell proliferation. This is biologically relevant as infection by *P. aeruginosa* occurs before the immune system is activated.

Future studies on the effect of QSSMs on Erk1/2 activation may benefit from using the ELISA technique to obtain a more quantitative analysis. The use of U0126, which is capable of directly inhibiting activated MEK1 and preventing endogenously active MEK 1/2 from phosphorylating and activating Erk 1/2 (Favata *et al.*, 1998, DeSilva *et al.*, 1998), represents a good negative control to this study.

Unpublished data from the immune modulation group at the University of Nottingham used FastKinase^{TM} profiling to assess the effects of 3-oxo-C12-HSL and PQS on T cell kinase activity. Results suggested 3-oxo-C12-HSL is not a kinase inhibitor while PQS significantly inhibited TrKA and Erk 1 activity. This suggests *P. aeruginosa* is able to inhibit additional pathways that may aid the host's immune system to defend against an infection, allowing the bacteria to maintain the infection.

To conclude, even though results here were inconclusive, this Ras pathway remains to be an important factor involved in the pathogenesis of *P. aeruginosa*, and where further research is essential.

6. Gene array analysis of the differential effects of QSSMs and CsA on RNA expression

6.1. Introduction

The previous chapters have focussed on looking at the effects of 3-oxo-C12-HSL and PQS on T cells. It was established that these two QSSMs suppress T cell proliferation and further cytokine and kinase analysis was performed to try to establish their mode of action. The aim of this chapter was to further explore possible targets of these QSSMs using gene array analysis.

Genome-wide monitoring of gene expression using DNA microarrays represents one of the latest break-through in experimental molecular biology and provides unprecedented opportunity to explore changes in cell signalling when faced with bacterial infections by providing a comprehensive survey of a cell's transcriptional landscape.

Using microarray analysis will help elucidate the pathways and entities responsible for the immune-modulatory effects of QSSMs and clarify molecular receptors involved in the process.

The concentrations of 3-oxo-C12-HSL and PQS used in this analysis were the IC_{50} concentrations extracted from the pure T cell proliferation assays.

6.2. Methods

Initially, after extraction to total RNA from QSSM-treated T cells, the Ambion Amino Allyl MessageAmp[TM] aRNA amplification kit was used following the protocol for two rounds of amplification to allow for dye incorporation and microarray analysis (section 5.2.2.). However, after several attempts to amplify the RNA to the appropriate concentration were unsuccessful, an alternative method was used for RNA amplification and dye incorporation – Agilents Low RNA Input Linear Amplification Kit (section 5.2.3.).

6.2.1. T cell stimulation, total RNA extraction and evaluation

T cells were isolated and cell density determined. 500 μl of cell suspension was added at a density of 10^6 cells per well of a 48-well tissue culture plates using CTCM. Cells were stabilised by incubating for 24 hr at 37°C in 5% CO_2. 19 μM 3-oxo-C12-HSL, 4 μM PQS and 3 μM CsA were tested with 0.1 μg/ml anti-CD3 and 5 μg/ml anti-CD28 bringing the final volume up to 1 ml per well with CTCM. Stimulated control samples (T cells stimulated with anti-CD3 and anti-CD28) and resting control samples (no stimulation) were included in this assay. The culture was incubated for 8 hr followed by suspending the stimulated T cells in RNA*later* RNA Stabilization Reagent (Qiagen, Valencia, CA, USA). RNA*later* Stabilization Reagent represents a novel technology for RNA protection by rapidly permeating tissues and single cells to stabilize and protect cellular RNA *in situ*. Briefly, cell suspensions were collected into eppendorfs and centrifuged at 300 x *g* for 5 min. Supernatants were removed completely and pellets washed by re-suspending in

500 μl PBS and spinning at 300 x *g* for 5 min.   Supernatants were removed completely and pellet re-suspended thoroughly in 100 μl PBS before further adding 500 μl RNA*later*.

RNeasy protect mini kit (Qiagen, Valencia, CA, USA) was used to isolate total RNA. This technology combines the selective binding properties of a silica-gel-based membrane with the speed of microspin technology. Briefly, the cell suspension was centrifuged at 3000 x *g* for 10 min and supernatant removed completely. The cell pellet was thoroughly re-suspended by flicking the eppendorfs. To lyse the cells, 350 μl of RLT buffer (containing 10 μl 2-ME per ml of RLT buffer) was added per tube and mixed via vortexing followed by the addition of 350 μl 70% ethanol (30% DEPC water (Ambion Ltd, Europe), 70% ethanol (Sigma, Poole, UK)) per tube before mixing via pipetting. The lysed cell mixture was then pipetted into an RNeasy mini column placed in a 2 ml collection tube. Samples were centrifuged at 8000 x *g* for 15 s, discarding the flow-through after and re-using the collection tube. The next step is the removal of contaminating genomic DNA to ensure that the specific PCR products are derived only from cDNA, and to avoid competition between the cDNA and genomic DNA during PCR reactions. Residual DNA was removed using the RNase-Free DNase I set (Qiagen, Valencia, CA, USA). DNase I stock solution was prepared by dissolving the solid DNase I (1500 Kunitz units) in 550 μl RNase-free water and mixed gently by inverting the tube. 10 μl of this DNase I stock solution was then added to 70 μl RDD buffer and mixed gently by inverting the tube before pipetting directly onto the RNeasy silica-gel membrane and incubated for 15 min at RT. After incubation, the column was washed with 350 μl RW1 buffer and spinning for 15 s at 8000 x *g*. The column was then transferred to a new 2 ml

collection tube before pipetting 500 μl RPE buffer onto the RNeasy column and centrifuging for 15 s at 8000 x g. The flow through was discarded before adding a second 500 μl RPE buffer onto the column and centrifuging for 2 min at 8000 x g to dry the membrane. To further remove any buffer residue the column was transferred to a new 2 ml collection tube and centrifuged at 10 000 x g for 1 min. To elute, the RNeasy column was transferred to a new 1.5 ml collection tube before adding 40 μl RNase-free water added directly onto the silica-gel membrane and centrifuging for 1 min at 8000 x g. To obtain a higher total RNA concentration, the eluate was collected and pipetted onto the silica-gel membrane again and centrifuged for a further 1 min at 8000 x g.

The samples were placed on ice and the RNA concentration was determined by NanoDrop® ND-1000 UV-Vis spectrophotometer (Labtech. International, Sussex, UK). RNase-free water was used as a blank control before pipetting 1.5 μl of the RNA elute onto the pedestal and measuring the concentration. RNA samples with A_{260}/A_{280} ratio of 1.9 – 2.1 and A_{260}/A_{230} ratio of 1.8 – 2.1 was further checked for its quality using an Agilent 2100 bioanalyzer analysis system (Agilent Technologies, Cheshire, UK). RNA samples chosen for further work had two well-defined 18S and 28S ribosomal RNA peak with an RNA integrity >8. If needed, the RNA samples were further concentrated using the RNeasy minElute Cleanup Kit (Qiagen, Valencia, CA, USA). This cleanup procedure allows purification and concentration of RNA from minimal elution volumes. RNA samples were adjusted to 100 μl with RNase-free water before adding 350 μl Buffer RLT and 250 μl 100% ethanol (Sigma, Poole, UK) mixing thoroughly each time via pipetting. 700 μl RNA mix was pipetted into an RNeasy MinElute Spin column in 2 ml collection tube and

centrifuged for 15 s at ≥8000 x *g* before transferring the column to a new 2 ml collection tube. 500 µl Buffer RPE was added to the column and centrifuged for 15 s at 8000 x *g* discarding the flow through and reusing the collection tube. 500 µl of 80 % ethanol was added to the column then centrifuged for 2 min at 8000 x *g* to dry before transferring to a new 2 ml collection tube and centrifuging for a further 5 min at 10 000 x *g* leaving the spin column cap open. The flow through and collection tube were discarded and column transferred to a new 1.5 ml collection tube. To elute, 14 µl RNase-free water was added directly onto the centre of the membrane and centrifuged for 1 min at maximum speed resulting in a 12 µl volume eluate of RNA in RNase-free water. RNA concentration was determined using the NanoDrop spectrophotometer before diluting the RNA to a concentration of 500 ng in a volume of 8 µl RNase-free water.

6.2.2. RNA amplification and Dye incorporation

The Ambion Amino Allyl MessageAmp^{TM} II aRNA Amplification kit (Ambion Ltd, Europe) was used. The two round amplification protocol was followed. All reagents were provided in the kit unless otherwise stated. To thoroughly mix the RNA and additional reagents added, a method of pipetting 3 times, flicking the tube four times and centrifuging briefly to settle the RNA was used unless otherwise stated.

Reverse transcription to synthesise first strand DNA

Briefly, 10 µl of total RNA (1000 ng as recommended for two rounds of amplification) in high quality water (Fisher Scientific Ltd, UK) was placed into a

non-stick, sterile, RNase-free 0.5 ml tube (Ambion Ltd, Europe) along with 1 µl of

T7 Oligo (dT) primer. Nuclease-free water was used to bring the final concentration

up to 12 µl before vortexing briefly then centrifuging to collect the mixture at the

bottom of the tube. 0.5 µl of control RNA spikes (Ambion Ltd, Europe) was then

added to every 100 ng of RNA and incubated for 10 min at 70°C in a thermal cycler.

After 10 min, the samples were briefly centrifuged and placed on ice while the

reverse transcription master mix (A.1.8.1) was prepared at room temperature in a

nuclease-free tube. 8 µl of the reverse transcription master mix was then added to

each RNA sample, mixed thoroughly, and centrifuged briefly before incubating at

42°C for 2 hr in an air incubator. After 2 hr, the samples were briefly centrifuged

and placed on ice, immediately proceeding to second strand cDNA synthesis.

Second strand cDNA synthesis

The second strand master mix (A.1.8.2) was prepared on ice in a nuclease-free tube

and mixed well by gently vortexing and centrifuging. 80 µl of the second strand

master mix was added to each RNA sample and mixed thoroughly, centrifuging

briefly, and then incubated for 2 hr at 16°C in a thermal cycler. After 2 hr, the

samples were placed on ice.

cDNA purification

250 µl of cDNA binding buffer (cDNA binding buffer and 100% ethanol) was added

to each sample and mixed thoroughly followed by a quick spin. The mixture was

then passed through a cDNA filter cartridge in a wash tube, and centrifuged for

1 min at 10 000 x *g* discarding the flow-through and replacing the cDNA filter cartridge in the wash tube. 500 µl of wash buffer was then applied to each cDNA filter cartridge and centrifuged for 1 min at 10 000 x *g*, discarding the flow-through before spinning the cDNA filter cartridge for an additional minute. The filter cartridge was then transferred to a cDNA elution tube. 9 µl of pre-heated nuclease-free water (55°C) was added to the centre of the filter then left at room temperature for 2 min before centrifuging for 2 min at 10 000 x *g*. The cDNA was eluted with a second 9 µl of pre-heated nuclease-free water, leaving the double-stranded cDNA in 14 µl of eluate.

In vitro transcription to synthesise biotin-labelled aRNA

The IVT master mix (A.1.8.3) was assembled at room temperature in a nuclease-free tube, mixed well via vortexing, and briefly centrifuged. 26 µl of this master mix was added to each sample mixing thoroughly before incubating at 37°C for 14 hr in a hybridization oven. After 14 hr, the reactions were stopped by adding 60 µl of nuclease-free water to each aRNA sample to bring the final concentration up to 100 µl.

aRNA purification using the Ambion amplification kit

This is required to remove unincorporated aaUTP and Tris from IVT reactions that would otherwise compete with the aRNA for de coupling; it also removes enzymes, salts, and other unincorporated nucleotides. 350 µl of aRNA binding buffer was

added to each sample before further adding 250 µl of ACS grade 100% ethanol (Sigma, Poole, UK) to each sample, mixing each sample by pipetting three times. Each sample was then pipetted onto the centre of the filter in the aRNA filter cartridge placed inside a collection tube and centrifuged for 1 min at 10 000 x g. The flow-through was discarded and the aRNA filter cartridge was placed back into the collection tube. 650 µl of wash buffer was then applied to each aRNA filter cartridge and centrifuged for 1 min at 10 000 x g before discarding the flow-through and spinning for a further minute. The filter cartridges were then transferred to a fresh aRNA collection tube. To the centre of the filter, 100 µl of pre-heated nuclease-free water (55^oC) was added and left at room temperature for 2 min before centrifuging for 2 min at 10 000 x g. The concentration of RNA was assessed using a nanodrop spectrophotometer before moving onto the second round of amplification.

Second round amplification

2 µg of purified aRNA was added to a sterile RNase-free microfuge tube (Ambion Ltd, Europe) along with 2 µl of second round primers, bringing the volume up to 12 µl with nuclease-free water. The samples were vortexed briefly and centrifuged before incubating for 10 min at 70^oC in a thermal cycler. After 10 min, the samples were centrifuged briefly and placed on ice before preparing the reverse transcription master mix for second round amplifications (A.1.8.1) at room temperature. 8 µl of this master mix was added to each sample, mixing thoroughly and centrifuging briefly before incubating for 2 hr at 42^oC in a thermal cycler. After 2 hr, 1 µl of RNase H was added to each sample, mixing thoroughly and centrifuging briefly, then incubated for 30 min at 37^oC in a thermal cycler.

Synthesis of second strand cDNA (second round)

After incubation, 5 µl of T7 Oligo(dT) primer was added to each sample vortexing to mix well and centrifuging briefly to collect the sample at the bottom of the tube. The samples were incubated for 10 min at 70°C in a thermal cycler and then placed on ice while preparing the second strand master mix for second round amplification (A.1.8.4). 74 µl of this master mix was added to each sample and mixed thoroughly before centrifuging briefly and incubating for 2 hr at 16°C in a thermal cycler. After 2 hr, the samples were placed on ice before moving onto cDNA purification, in vitro transcription to synthesise biotin-labelled aRNA and aRNA purification.

aRNA: dye coupling reaction

Once the aRNA concentration was determined, 5-20 µg aRNA was vacuum dried making sure not to over dry. In the meantime, 11 µl of 100% DMSO (Sigma, Poole, UK) was added to each tube of Cy3 and Cy5 reactive dyes (Amersham Biosciences, UK) and vortexed to mix. The dried aRNA was then resuspended in 9 µl of coupling buffer and vortexed gently. 11 µl of prepared dye was then added to each RNA sample, again vortexing gently to mix before incubating for 30 min at room temperature away from light. 4.5 µl of 4 M hydroxylamine was then added to each sample vortexing to mix and incubating for 15 min at room temperature away from light. 5.5 µl nuclease-free water was then added to each sample bring the final concentration up to 30 µl.

Dye labelled aRNA purification

105 μl aRNA binding buffer was added to each sample followed by 75 μl of 100% ethanol and mixed by pipetting up and down three times. Each sample was then pipetted into a labelled aRNA filter cartridge in a collection tube and centrifuged for 1 min at 10 000 x g. The flow-through was discarded and the filter replaced in the collection tube. 500 μl of wash buffer was then added into the filter and centrifuged for 1 min at 10 000 x g, again discarding the flow-through before spinning the filter for an additional minute. The labelled aRNA filter was then transferred to a labelled aRNA elution tube. 10 μl of pre-heated nuclease-free water (55$^{\text{o}}$C) was then added to the centre of the filter and left at room temperature for 2 min before centrifuging for 2 min at 10 000 x g. An additional 10 μl of pre-heated nuclease-free water was added to the centre of the filter and left for 2 min at room temperature before centrifuging for 2 min at 10 000 x g bringing the labelled aRNA volume to 20 μl with nuclease-free water.

6.2.3. Alternative method used for RNA amplification and dye incorporation

Agilent's Low RNA Input Linear Amplification kit (Agilent Technologies, Cheshire, UK) was used to amplify and label the total RNA obtained when amplification was unsuccessful using the Ambion kit. This Agilent's amplification kit generates fluorescent complimentary RNA (cRNA).

Briefly, to synthesise cDNA from total RNA, 500 ng of total RNA in a volume of 8 μl in RNase-free water was added to a 0.2 ml PCR tube with 1.2 μl of T7 promotor primer and 2.5 μl of two-colour spike-in mix. The tubes were placed in a thermo cycler and incubated for 10 min at 65°C to denature the template and anneal the primer, then cooled at 4°C. Immediately before use, the cDNA master mix was prepared by mixing 4 μl 5x first strand buffer, 2 μl 0.1 M DTT, 1 μl 10mM dNTP mix, 1 μl MMLV-RT and 0.5 μl RNase OUT then transferred to the RNA mix, pipetting up and down to mix before incubating in the thermo cycler at 40°C for 2 hr then 65°C for 15 min to inactivate the MMLV-RT and finally cooled at 4°C. The samples were then spun down briefly to drive the contents to the bottom of the tube.

The next step is *in vitro* transcription and incorporation of cyanine 3-CTP (cy3) and cyanine 5-CTP (cy5) (Agilent Technologies, Cheshire, UK). Briefly, the transcription master mix was prepared by mixing 15.3 μl nuclease-free water, 20 μl 4x transcription buffer, 6 μl 0.1 M DTT, 8 μl NTP mix, 64 μl 50 % PEG, 0.5 μl RNase OUT, 0.6 μl Inorganic pyrophosphatase, 0.8 μl T7 RNA polymerase, 2.4 μl cy3 and 2.4 μl cy5 together. To each sample tube, 60 μl of the transcription master mix was added and gently mixed via pipetting before incubated in a thermal cycler for 2 hr at 40°C then cooled at 4°C.

Purification of Amplified RNA

Qiagen's RNeasy mini spin columns (Qiagen, Valencia, CA, USA) were used to purify the amplified cRNA samples. Unincorporated dye-labelled nucleotide in the hybridization buffer solution significantly increased background fluorescence on the

microarray. All centrifugations used in this protocol were carried out at 4°C to obtain higher yields. 20 μl of nuclease-free water was added to the cRNA sample to obtain a total volume of 100 μl. To this, 350 μl of Buffer RLT was added and mixed well before adding 250 μl of 100% ethanol mixing well via pipetting. 700 μl of this cRNA sample was transferred to an RNeasy mini column in a 2 ml collection tube and centrifuged at 10 000 x g for 30 s discarding the flow through and collection tube after. The column was then transferred to a new 2 ml collection tube before adding 500 μl of Buffer RPE to the column and centrifuging at 10 000 x g for 60 s. The flow-through was discarded and an additional 500 μl of Buffer RPE was added to the column and again spun at 10 000 x g for 60 s. To elute the now cleaned cRNA sample, the column was transferred to a new 1.5 ml collection tube before adding 30 μl RNase-free water directly onto the filter membrane and waiting 60 s prior to centrifuging for 30 s at 10 000 x g. The RNeasy column was discarded and the cRNA sample retained in 30 μl of RNase-free water.

6.2.4. Quantitating cRNA products

cRNA was quantitated using a ND-1000 spectrophotometer (Labtech International, Sussex, UK) to minimise the amount of sample consumed by the measurement. The microarray tab was used to scan the appropriate wavelengths. 1.5 μl of nuclease-free water was used to blank the nanodrop instrument then 1.5 μl of amplified cRNA was used for analysis. The concentration of cRNA was calculated by using the following formula:

$$\text{Conc. of cRNA (μg/μl)} = OD_{260} \times 10 \times 40 \text{ μg/ml)}/100$$

The reaction is multiplied by 10 because the path length of analysis is 1mm. To calculate the specific activity of cRNA the following formula was used:

Specific Activity = pmol dye per µl/ conc. of cRNA (µg/µl)

Specific activity from reactions starting with 500ng of input RNA was between 10-15 pmol/µg. Any cRNAs with specific activities less than 8 pmol/µg were not used as microarray targets.

6.3. Results

6.3.1. Assessment of RNA quantity using a nanodrop spectrophotometer

After initial isolation of total RNA from pure human T cells under control and testing conditions, RNA concentration was assessed using nanodrop spectrophotometry. Concentrations ranged from 190 to 250 ng/µl (data not shown). To increase the RNA concentration to the amount required for PCR and dye incorporation (500 ng/µl), the RNA isolated for each condition was pulled together and further purified and concentrated using the RNeasy minElute Cleanup Kit. Final concentrations obtained for each testing and control conditions were adjusted to 500 ng/µl (data not shown).

6.3.2. Assessment of RNA quality using Agilent 2100 Bioanalyzer

The quality of RNA obtained was assessed using the bioanalyzer. Results indicate good quality RNA was isolated with no degradation to each sample isolated. RNA integrity number (RIN) ranged from 8 to 10 (fig. 6.1 and 6.2).

Figure 6.1
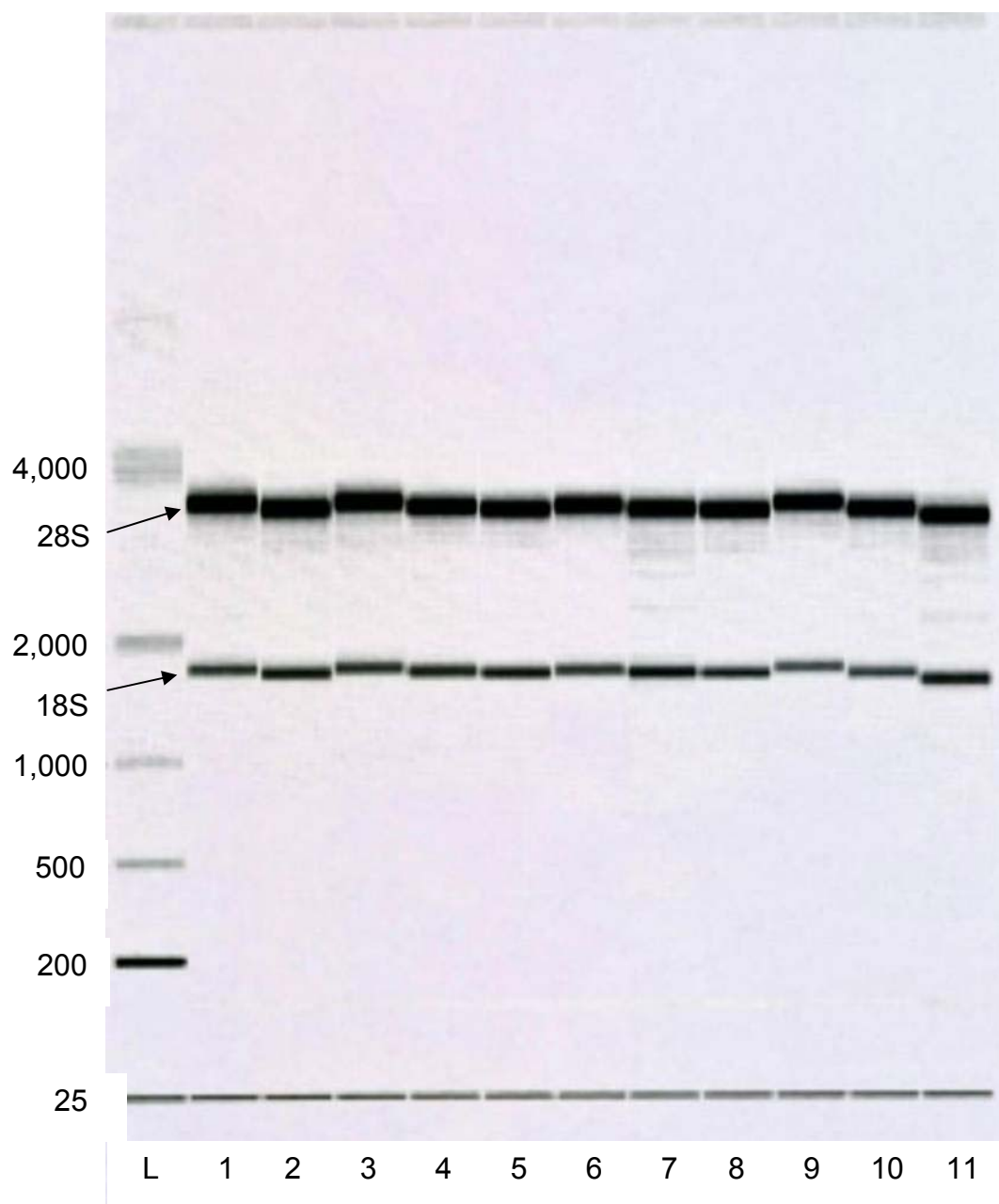
Figure 6.1. Gel image from bioanalyzer showing the 18S and 28S RNA bands.

The first lane (L) represents the reference ladder, lane (1) pooled stimulated control, lane (2) individual stimulated control, (3) resting control, (4) 3-oxo-C12-HSL-treated RNA, (5) PQS-treated RNA, (6) CsA-treated RNA, lanes 7 to 11 are repeats of lanes 2 to 6.

Figure 6.2
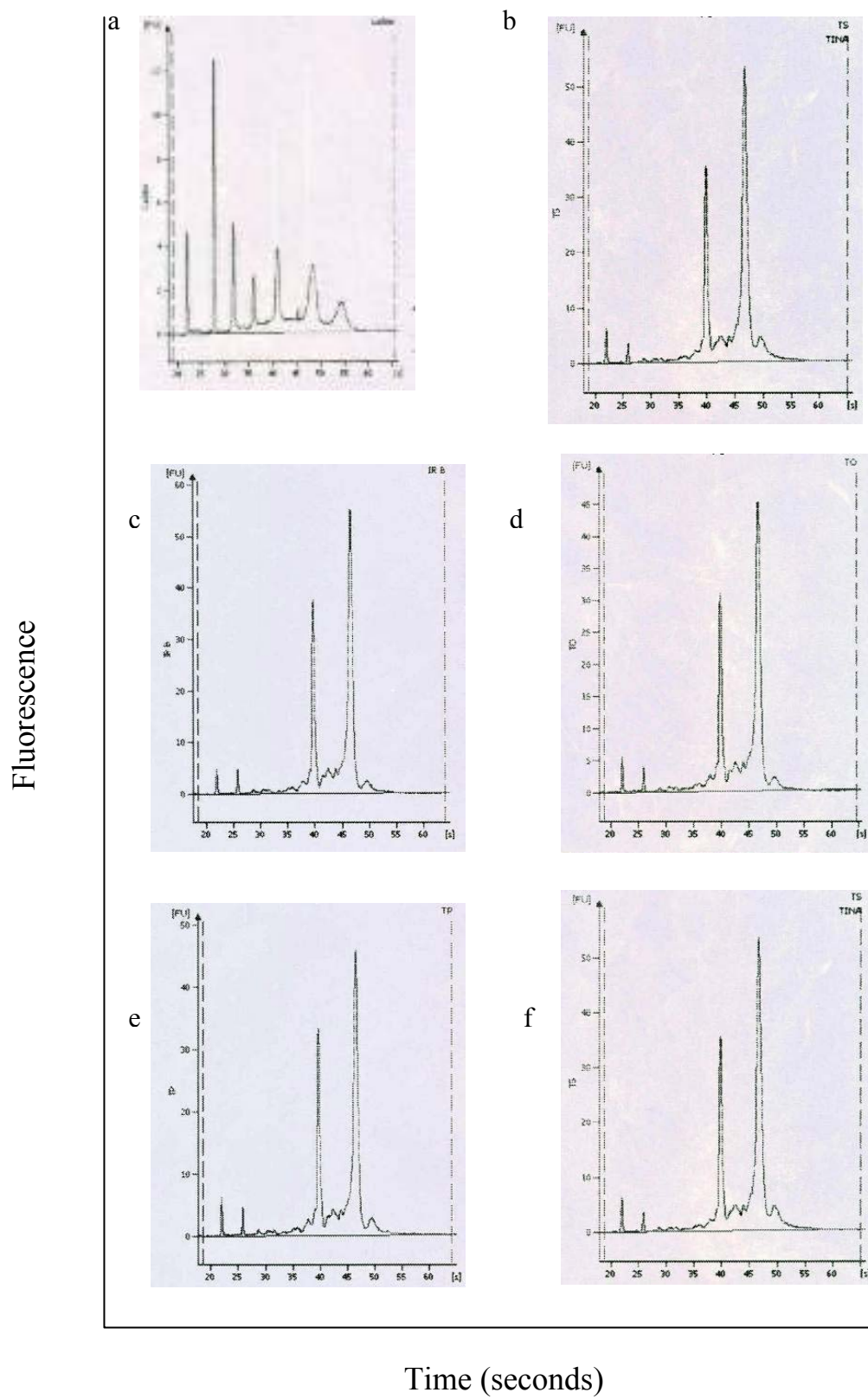
Figure 6.2. Electropherogram summaries of the RNAs isolated from each test sample. Graph (a) shows the ladder reference, (b) stimulated control i.e. cells treated only with anti-CD3 and anti-CD28, (c) resting control i.e. untreated cells, (d) cells stimulated with 24 μM 3-oxo-C12-HSL, (e) cells stimulated with 4 μM PQS and (f) cells stimulated with 3 μM CsA. Intact total RNA was isolated. The peaks at 40 s and 45 s represents 18S and 28S RNA respectively.

6.3.3. Summary of Microarray work

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After isolation of good quality RNA for pure T cells, amplification of the RNA to the required concentration was not successful and so the intention to perform gene array analysis was not wholly successful on this occasion. However, in parallel to this study, gene array analysis performed to detect differential gene expression levels of stimulated T cells treated with PQS was completed by more experienced experts at Mars Inc. Their findings will be further detailed in the discussion.

6.4. Discussion

Intact total RNA was obtained in the concentration required. The Ambion amplification kit was initially used following the protocol for two rounds of amplification. The starting concentration used was 100 ng/ml as recommended by Ambion Ltd. However, after performing the PCR process, the concentration of RNA never reached the appropriate concentration of 5 μg required for the dye coupling reaction. After repeating the amplification process several times with no success using the Ambion reagents provided in the kit, and performing repeat checks on the RNA integrity using the bioanalyzer, advice was given to research into a second amplification kit. The Agilents' Low RNA Input Linear Amplification kit (Agilent Technologies, Cheshire, UK) was recommended. After comparing both the Ambion and Agilent protocol, it was acknowledged that the Agilent low RNA input Linear amplification kit was optimized to generate fluorescent complimentary RNA (cRNA) for use with Agilent's oligonucleotide microarrays, where as the Ambion Allyl MessageAmp II aRNA (anti-sense RNA) Amplification Kit was not specifically made for Agilent microarrays use.

In hindsight, it may have been more appropriate to use the Agilent kit as the microarray facilities that would have been used is from Agilent Technologies. The concentration of aRNA required for the dye coupling reaction for the Ambion kit was also extremely high compared with the Agilent kit (5000 ng and 50 ng respectively). This lead to question the reason why the Ambion kit required such high concentrations of aRNA for the dye coupling reactions. Explanations for this could be that the efficiency of labelling is better with the Agilent kit compared with the

Ambion kit where it may have been limited to how much dye can be bound to the aRNA samples.

The Immune Modulation group at the University of Nottingham, where this present study was performed work in collaboration with Mars Inc. In parallel to this microarray study, members of this research group also sent samples of stimulated T cells treated with PQS and T cells only stimulated to proliferate for external microarray analysis, to experienced experts at Mars Inc. These findings are summarised in table 6.1 and table 6.2, showing changes in gene expression relating to immune function.

It was observed that the activation of T cells by anti-CD3 and anti-CD28 increased gene expression of cytotoxic T-lymphocyte associated protein 4 (CTLA4, a member of the immunoglobulin superfamily and encodes a protein which transmits an inhibitory signal to T cells), IFNγ (a hallmark cytokine for Th1 cells), and IL-2 receptor alpha (IL-2Rα, associated with constituting the high affinity IL-2 receptor) by ten fold. The increase in IL-2Rα is an important factor as T cell activation is associated with IL-2 production. An increase in the IL-7 receptor gene was also detected, important for proliferation during certain stages of T cell survival, development and homeostasis.

When T cells were stimulated with anti-CD3 and anti-CD28 in the presence of PQS, the microarray results indicated a 1.6 fold decrease in IL-12B (Table 6.2). This gene encodes a subunit of IL-12, a cytokine that acts on T and natural killer cells, as well as being responsible for IFNγ biosynthesis and the positive regulation of activated

T cell proliferation. This cytokine is expressed by activated macrophages that serve as an essential inducer of Th1 cells development. This cytokine has been found to be important for sustaining a sufficient number of memory/effector Th1 cells to mediate long-term protection to an intracellular pathogen. A decrease in IL-12B may therefore indicate PQS influences T cell function by suppressing Th1 response due to the inhibition of IFNγ biosynthesis and suppressing the positive regulation of activated T cell proliferation this gene has. Despite this, the introduction of PQS to T cells will still initiate an increase in genes responsible for launching an immune response (Table 6.2), most noticeably CD7 antigen (p41) which encodes a transmembrane protein found on thymocytes and mature T cells and plays an essential role in T cell interactions, and IFNα-inducible protein (clone IFI-6-16) which helps launch immune responses to pest, pathogen or parasites.

In conclusion, although unsuccessful in performing the microarray analysis myself, collaboration with Mars Inc. has allowed me to gain insight into the effects PQS may have on gene expression. PQS has shown to inhibit IL-12B suggesting suppression of Th1 response and furthering our understanding of immune modulation by PQS.

Table 6.1. Differential gene expression levels of stimulated T cells. Summary of changes in gene activation that are involved in immune response.

10 Fold Change Increase

Description	Biological Process
Cytotoxic T-lymphocyte-associated protein 4	Immune response
Interferon, gamma	Immune response
Interleukin 2 receptor, alpha	Regulation of cell cycle, apoptosis
Nedd4 family interacting protein 2	Positive regulation of NF-kappaB cascade
Suppressor of cytokine signalling 3	Regulation of intracellular signalling cascade, JAK-STAT cascade, negative regulation of signal transduction

8 Fold Change Increase

Description	Biological Process
TNF superfamily, member 2	Regulation of transcription, apoptosis, anti-anti-apoptosis

6 Fold Change Increase

Description	Biological Process
IL-7 receptor	Immune response, cell surface receptor linked signal transduction

6 Fold Change Decrease

Description	Biological Process
C-type lectin domain family 4, member A	Immune response, cell surface receptor linked signal transduction
CDC28 protein kinase regulatory subunit 2	Cell proliferation
S100 calcium binding protein A12 (calgranulin C)	Defence response to bacteria

Table 6.2. Differential gene expression levels of stimulated T cells treated with

PQS. Summary of changes in gene activation that are involved in immune response.

1.6 Fold Change Increase

Description	Biological Process
2',5'-oligoadenylate synthetase 1, 40/46 kDa	Immune response, response to virus
IFN, α-inducible protein (clone IFI-15K)	Immune response, cell-cell signalling
IFN, α-inducible protein (clone IFI-6-16)	Immune response, response to pest, pathogen or parasite
Myxovirus (influenza virus) resistance 1, IFN-inducible protein p78 (mouse)	Immune response, response to virus
Myxovirus (influenza virus) resistance 2 (mouse)	Immune response, response to virus

1.6 Fold Change Decrease

Description	Biological Process
IL-12B	Antimicrobial humoral response, T-helper cell differentiation, IFNγ biosynthesis, positive regulation of activated T cell proliferation

1.5 Fold Change Increase

Description	Biological Process
2',5'-oligoadenylate synthetase 2, 69/71 kDa	Immune response
CD7 antigen (p41)	Cellular defence response, T cell activation
RPA interacting protein	Immune response

7. General Discussion

P. aeruginosa is an opportunistic pathogen of immunocompromised patients indicating that it is often vulnerable to the human immune system. Quorum sensing (QS) is a mechanism whereby bacteria are able to sense the environment and, as a population, regulate the expression of various genes, including those important for the virulence of the organism, and are involved in elevated antibiotic tolerance of biofilms as well as elevated tolerance to activity of the innate immune system. Therefore, the bacteria in a joint effort are able to subvert the host defences and cause infections. In *P. aeruginosa*, QS regulates expression of numerous virulence genes that are thought to be important for the pathogenesis of the organism.

Recent progress in QS research has demonstrated that *Pseudomonas* QSSMs are not only important in the regulation of bacterial virulence genes but also interact with eukaryotic cells and modulate immune responses (Dimango *et al.*, 1995, Telford *et al.*, 1998, Saleh *et al.*, 1999), thus the QS signalling system represent highly attractive targets for the development of novel therapeutics. Given the detection of functional *P. aeruginosa* QSSMs in sputum and biopsy material from patients with cystic fibrosis (Singh *et al.*, 2000, Collier *et al.*, 2002, Favre-Bonte *et al.*, 2002, Middleton *et al.*, 2002, Chambers *et al.*, 2005), their role in biofilm establishment and maintenance, and their implication of driving the disease process associated with infection (Wu *et al.*, 2000, Donabedian, 2003, Bjarnsholt *et al.*, 2005a), it was of interest to assess the immune modulatory capability of these QSSMs.

Early research has already indicated 3-oxo-C12-HSL, but not C4-HSL or 3-oxo-C6-HSL, suppresses IL-12 and TNF-α secretion by bacterial lipopolysaccharide (LPS)-stimulated macrophages (Telford *et al.*, 1998), and more recently, Smith *et al.* (2002) reported 3-oxo-C12-HSL induces cyclooxygenase-2 and prostaglandin E_2 production in lung fibroblasts suggesting a pivotal role for 3-oxo-C12-HSL in inflammation. Presently, Miyairi *et al.* (2006) demonstrated that specific antibodies to 3-oxo-C12-HSL play a protective role in acute pulmonary *P. aeruginosa* infections, and that the bacterial QS system, especially autoinducer molecules, to be a novel candidate for vaccine development against *P. aeruginosa* infection (Miyairi *et al.*, 2006). Previous studies suggest T cells play an important role in defending the host against the attack of *P. aeruginosa*, for example, Stevenson *et al.* have shown BALB/c mice are resistant to the establishment of chronic pulmonary *P. aeruginosa* infections and mount significantly enhanced T cell response to the mitogen ConA (Stevenson *et al.*, 1995). Suppression of T cell activity is therefore likely to be advantageous to *P. aeruginosa* and warrants further research into how *P. aeruginosa* manipulates human T cell function.

Differential immune-modulatory activity of *P. aeruginosa* QSSMs has been previously described (Pritchard *et al.*, 2003, Hooi *et al.*, 2004) citing the ability to promote bacterial communication while suppressing the immune system. Hooi *et al.* (2004) demonstrated that the two structurally diverse QSSMs, 3-oxo-C12-HSL and PQS, could exert differential modulatory effects on mammalian immune responses more specifically peripheral blood mononuclear cells (PBMCs) *in vitro*. Differential immune-modulatory activity was observed when TNF-α secretion was assessed in assays where LPS were used to drive TNF-α secretion from hPBMC, with

3-oxo-C12-HSL (suppressive) and PQS (stimulatory) exhibiting contrasting effects (Hooi *et al.*, 2004). The same effects was also demonstrated with IL-2 release from αCD3 and αCD28 driven hPBMCs (Hooi *et al.*, 2004). It has been reported that the *in vitro* effects of 3-oxo-C12-HSL and PQS on lymphocytes are suppressive, including inhibition of proliferation (Chhabra *et al.*, 2003, Hooi *et al.*, 2004) and cytokine production (Telford *et al.*, 1998, Smith *et al.*, 2001, Ritchie *et al.*, 2003). So with this knowledge, this study focused on the immunomodulatory effects of 3-oxo-C12-HSL and PQS on increasingly pure T cell populations (chapter 3), as T cells have been shown to be important to immunity against *P. aeruginosa* (Stevenson *et al.*, 1995). This present study has confirmed the immunosuppressive properties of 3-oxo-C12-HSL and PQS in mouse splenocytes and hPBMCs, and has shown for the first time their inhibitory effect on pure human T cells. CsA, as the positive control, has potent immunosuppressive properties with potency similar to PQS in pure T cells (chapter 3 and 4).

Very recently Bredenbruch *et al.* (2006) provided novel insight into the molecular mechanisms underlying PQS signalling and demonstrates that well documented effects of PQS signalling can at least in part be traced back to its activity as an iron chelator (Bredenbruch *et al.*, 2006). As a consequence of its iron-chelating activity, the transcriptional profile of PAO1 in response to PQS reflects an iron starvation response, accompanied by the up-regulation of genes required for iron acquisition, some of which was shown to be regulated by QS (Bredenbruch *et al.*, 2006). Bredenbruch *et al.* (2006) also demonstrated *rhl* QS system was activated under iron-limiting conditions thus affects *rhl* QS system indirectly as a result of iron depletion. Finding that PQS influences the *P. aeruginosa* iron haemostasis is an

important step in the understanding of complex bacterial interactions and endorses the growing evidence that cell-to-cell signalling mediated by QS system can also be strongly affected by environmental factors other than cell density. Moreover, the PQS precursor 4-hydroxy-alkyl-quinoline has recently been described to exhibit iron-chelating activities although it is not confirmed using synthesized compound (Royt et al., 2001). The consequences of PQS as a potent iron-chelator means when added to growth medium, PQS depletes iron from the medium so that the bacteria experience iron deprivation and react with an increased expression of iron acquisition systems, PQS production and *rhl* expression. This questions the extent to which PQS as an iron chelator may affect T cell response to this compound, whether this may be accountable for the high potency obtained for PQS compared to 3-oxo-C12-HSL. Further work is therefore required to assess the extent PQS' iron chelating property affects cell culture growth, if any.

Although unable to speculate the clinical relevance of the inhibitory effects of the two QSSMs, it is possible to hypothesise that 3-oxo-C12-HSL and PQS may, in the future, be relevant in providing alternative immunosuppressive agents either in conjunction with or in place of present immunosuppressants such as CsA, FK506 and rapamycin. CsA was originally isolated from cultures of the fungus *Tolypocladium inflatum*, while rapamycin is a natural product derived from a soil micro-organism *Streptomyces hygroscopicus*. CsA, FK506 and rapamycin have not only revolutionised the field of organ transplantation but have also been used as a molecular tools for dissecting intracellular signal transduction pathways involved in T cell activation (Schreiber, 1991, Schreiber, 1992). Even though this present study illustrates the potency of CsA to be higher than 3-oxo-C12-HSL and PQS, the side

effects of CsA therapy are the greatest limitation to wider use of this drug, particularly in autoimmune diseases where prolonged administration may be anticipated. In general, the toxicity of CsA can be divided into two groups; (a) side effects associated with inhibition of immune responsiveness and, therefore, shared with other non-specific immunosuppressive agents; and (b) toxicities in other organ systems that are unique to this class of compounds. The latter category is the more clinically troublesome, with renal dysfunction the most frequent serious complication of CsA therapy (Kahan, 1989).

Other immunosuppressive drugs such as FK-506 and rapamycin are also limited in use due to undesirable side effects. FK-506, although chemically unrelated to CsA affect a similar subset of calcium-associated signalling events involved in the regulation of lymphokine gene expression, activation-driven T cell death and exocytosis. The most common acute side effects reported mirror those observed with CsA; neurologic (headache, insomnia, tremors, paresthesias, lethargy); gastrointestinal (nausea, vomiting, diarrhoea); renal (increase in blood urea nitrogen and creatinine); and diabetes (Kahan, 1989, Shapiro *et al.*, 1990). However, in general, renal dysfunction appears to be less severe with FK-506 than with CsA, and the frequency with which patients treated with FK-506 receive anti-hypertensive medication is significantly less than that of CsA-treated patients.

Rapamycin has structural similarity with FK-506 but suppresses T cell activation at a different level, mainly through inhibition of proliferation induced by growth promoting lymphokine, with molecular targets including RAF T1/FRAP proteins in mammalian cells associated with cell cycle progression through G_1 phase (Chiu *et*

al., 1994, Sabatini *et al.*, 1994, Almawi *et al.*, 1999, Gingras *et al.*, 2001). Its main advantage is that it appears to lack significant nephrotoxicity, where lymphocele, hypercholesterolemia, hypertriglyceridaemia, peripheral oedema, leukopenia and thrombocytopenia are the most common adverse effects identified in clinical studies. However, as consequence of its inability to interfere with early events after T cell activation, rapamycin is a less effective inhibitor of cytokine synthesis than CsA and FK-506. The benefits of present immunosuppressants are therefore limited by their side effects and so alternative drugs are constantly being pursued. The similar effects of 3-oxo-C12-HSL and PQS to CsA and rapamycin makes them potential targets for alternative drugs and so requires a more in depth study to their mode of action.

The potent immunosuppressive properties of CsA are reflected by its ability to block the transcription of cytokine genes in activated T cell, including those of IL-2 and IL-4 (Kronke *et al.*, 1984, Herold *et al.*, 1986, Granelli-Piperno, 1988). Previous reports have suggested 3-oxo-C12-HSL and CsA have similar immunosuppressive properties (Hooi *et al.*, 2004), so to further understand the molecular mechanisms of action of the two QSSMs, the effects of 3-oxo-C12-HSL and PQS on cytokine production in pure T cells was investigated (chapter 4).

Cytokines are proteins that play an integral role in the human immune response. The functions of these proteins are diverse and include roles in normal T cell-mediated immunity, the inflammatory response, cancer, auto immunity, and allergy (Borish and Rosenwasser, 1996). Therefore, various pathologic conditions will be accompanied by changes in cytokine levels. The cytokines measured include

Th1-cell cytokines (IFNγ and TNFβ), Th2-cell cytokines (IL-4 and IL-5), lymphocyte cytokines (TNFα and IL-10), and a cytokine produced by both Th1 and Th2 cells (IL-2) (Borish and Rosenwasser, 1996).

Activation of T cells through the TCR is followed by a cascade of intracellular signalling events, culminating in the transition from the G_0 to G_1 phase of the cell cycle and the induction of cytokine gene expression leading to secretion of IL-2 and surface experiments of the IL-2 receptor (IL-2R). The interaction of IL-2 with the IL-2R initiates a second phosphorylation cascade and sequential wave of gene expression that drives the S phase followed by clonal expansion (Smith, 1988, Crabtree, 1989).

This present study demonstrated that 3-oxo-C12-HSL inhibited both T cell proliferation and IL-2 production supporting the finding that 3-oxo-C12-HSL acts on early T cell events (Ritchie *et al.*, 2005). In contrast, PQS inhibited T cell proliferation while enhancing IL-2 release indicating a cytostatic effect and supporting previous suggestions that PQS acts proximally to the IL-2R or downstream of the IL-2R in the T cell signalling pathway. These activities are reminiscent of cyclosporin A and rapamycin respectively. CsA and rapamycin are microbial products with potent immunosuppressive properties that result primarily from a selective inhibition of T lymphocyte activation, where CsA prevents the transcription of early T cell activation genes (Kronke *et al.*, 1984) and rapamycin inhibits proliferation in response to growth factors such as IL-2 (Hatfield *et al.*, 1992, Quesniaux *et al.*, 1994). CsA and rapamycin have become useful probes in

understanding lymphocyte signal transduction and in uncovering the patho-physiologic processes involved in certain clinical situations.

In addition, the inhibition of IFNγ by both QSSMs suggests the suppression of T cell activation is via Th1 as IFNγ is required for Th1 activation. Furthermore, 3-oxo-C12-HSL also inhibited IL-4 release while PQS inhibited IL-4 release only at 3.12 μM. The inhibition of IL-4 release by 3-oxo-C12-HSL may indicate that this QSSM may also inhibit T cell function by inhibition of Th2, as IL-4 is required to stimulate Th2 activity. Telford *et al.* (1998) reported that 3-oxo-C12-HSL inhibits the production of IL-12 from activated monocytes. Since IL-12 is a potent stimulator of T cells to produce IFNγ, it was hypothesised that its inhibition by 3-oxo-C12-HSL would lead to a decrease in T cells with the Th1 phenotype and therefore induces a Th2 phenotype.

In contrast, Smith *et al.* (2002) suggested that 3-oxo-C12-HSL directly interacts with T cells to produce IFNγ in the absence of IL-12 production, therefore proposed that 3-oxo-C12-HSL is able to modulate the immune response during *P. aeruginosa* infections by stimulating the inflammatory Th1 phenotype not Th2 response. However, the two studies are not totally contradictory as the effects observed by Smith *et al.* (2002) tended to work with high doses of up to 100 μM compared to the low doses (<10 μM) used by Telford *et al.* (1998). In addition, high doses of 3-oxo-C12-HSL (similar to the concentrations used by Smith *et al.* (2002) have been confirmed to cause apoptosis in eukaryotic cells (Pritchard *et al.*, 2003, Tateda *et al.*, 2003), thus questioning the validity of experiments conducted at high doses of 3-oxo-C12-HSL.

Further studies by Ritchie *et al.* (2005) analysed the effects of 3-oxo-C12-HSL on primary and secondary stimulation of CD4 T cells strongly rather than selectively inhibits the development of either Th1 or Th2 cells, 3-oxo-C12-HSL inhibits the development of all effector CD4 T cells. However, a possible explanation for the reports of preferential effects of 3-oxo-C12-HSL on either Th1 (Smith *et al.*, 2002) or Th2 (Telford *et al.*, 1998) responses can be explained from two pieces of data. Firstly, observations that in secondary stimulation, IFNγ production appears to be more sensitive to 3-oxo-C12-HSL than does IL-4, this means that in secondary responses, a preferential development of Th2 responses could occur, and 3-oxo-C12-HSL treatment could favour a Th2-like bias for a long-term response. Secondly, the fact that the affinity of the antigen-TCR interaction modulates the outcome of 3-oxo-C12-HSL inhibition means that the overall effect of 3-oxo-C12-HSL on a response could depend on the affinity of the antigen in question for the relevant TCR.

Additionally, microarray analysis indicated a decrease in the gene IL-12B when T cells were treated with PQS. As mentioned in chapter 5, this gene is responsible for the biosynthesis of IFNγ that serve as an essential inducer of Th1 cells development. A decrease of the gene IL-12B therefore indicates PQS may suppress Th1 response, confirming results obtained from cytokine analysis.

To further understand the immune-modulation of these QSSMs, possible molecular targets of 3-oxo-C12-HSL and PQS within the T cell/IL-2 signalling pathway was considered. Smith *et al.* (2001) demonstrated a specific signal pathway that was stimulated by 3-oxo-C12-HSL in bronchial epithelial cells. 3-oxo-C12-HSL

activated extracellular signal regulated kinases (Erks) that subsequently induced the activation of the transcription factor NFκB. This activation of NFκB was essential for maximal production of IL-8 with 3-oxo-C12-HSL stimulation (Smith *et al.*, 2001).

The broad range of effects of 3-oxo-C12-HSL on different types of mammalian cells, combined with the range of immunomodulatory effects reported for 3-oxo-C12-HSL, suggest that it is most probably acting either through a specific target that is ubiquitous to many cell types, such as NFκB or an NFκB-dependent pathway, or that 3-oxo-C12-HSL is in fact interacting with a range of different targets.

With respect to the hypothesis that 3-oxo-C12-HSL and PQS affect different IL-2 pathways, this study aimed to observe whether the Erk pathway is directly inhibited by the QSSMs. Results in this study were inconclusive to the effects both QSSMs may have on Erk 1/2 activity (chapter 5), however, 3-oxo-C12-HSL has already been implicated in the NFκB pathway where it has been shown to activate NFκB and AP-2 resulting in production of IL-8 (Smith *et al.*, 2001). Smith *et al.* (2001) also demonstrated 3-oxo-C12-HSL is able to specifically up-regulate both Erk1 and 2 in the cytoplasm of 16HBE cells. Research within the immune-modulation group at the University of Nottingham has also indicated PQS inhibits TrKA activity leading to subsequent suppression in Erk 1 production (unpublished data).

7.1. Future Work

Additional molecular targets of these two QSSMs that warrant future research are peroxysomes proliferator activated receptors (PPARs). PPARs are members of the hormone receptor superfamily which are ligand-activated transcription factors regulating gene expression. 3-oxo-C12-HSL as a possible PPARγ agonist is currently under investigation. PPARγ has been shown to play a role in the differentiation and activation of monocytes and the regulation of inflammatory activities (Tontonoz *et al.*, 1998, Ricote *et al.*, 1999), and expression and function of PPARγ in T lymphocytes were recently demonstrated (Clark *et al.*, 2000). Clark *et al.* (2000) demonstrated 15 d-PGJ2 (a ligand of PPARγ) mediated significant inhibitions of the anti-CD3 antibody-stimulated proliferative responses of freshly isolated T cell enriched splenocytes (Clark *et al.*, 2000) . Yang *et al.* (2000) reported PPARγ is expressed in human peripheral blood T cells and demonstrated that the activated PPARγ physical associates with the transcription factor nuclear factor of activated T cells (NFAT) thus blocking its DNA binding and transcriptional activation of the IL-2 promoter. The activation and function of NFAT are known to be absolute requirements of IL-2 transcription (Yang *et al.*, 2000). The inhibitory effect on pure T cells and IL-2 by 3-oxo-C12-HSL may be in part due to its effect as a PPARγ agonist.

It may also be of interest to research into any effect 3-oxo-C12-HSL and PQS may have on Tregs. Previous research have shown rapamycin enhances the relative levels of $CD4^+CD25^+$ Tregs in the spleen and thymi of mice while most T cell subsets were decreased (Qu *et al.*, 2007). In contrast, CsA did not abrogate, but reduced the

function of allostimulated Treg cells *in vitro* (Zeiser *et al.*, 2006). Since 3-oxo-C12-HSL and PQS have been compared to immunosuppressive agents, their effect, if any, on Tregs may help advance our understanding on the way these QSSMs work.

Future study on the synergistic effects of 3-oxo-C12-HSL and PQS on T cell activation and proliferation is also of importance. Whether the combined effect of these two QSSMs is greater or indifferent or even differ to what we know, will contribute to the further understanding of the mechanisms of actions of 3-oxo-C12-HSL and PQS.

Continual research into the effects of 3-oxo-C12-HSL and PQS at an RNA level using microarray technology will also be important. Insight into the RNAs activated/changed will provide a useful tool in the future if these two QSSMs are to be considered for possible use as an immunosuppressant as it will provide detailed analysis of what pathways and proteins are affected by these QSSMs.

Very recently, work has been carried out to discover the molecular receptor for 3-oxo-C12-HSL using affinity chromatography. Actin and profilin were found to bind to [^{3}H]3-oxo-C12-HSL, and S100A8 (calgranulin A) and S100A9 (calgranulin B) was presented as a possible receptor for 3-oxo-C12-HSL. The continuation of this work especially on T cells is important for better understanding of the mode of action for this QSSM.

7.2. Conclusion

Bacterial communication via quorum sensing is an important component in the production of virulence factors, antibiotic sensitivity, and biofilm development. *P. aeruginosa* possesses one to the best studied model of quorum sensing which may contribute to the ability of *P. aeruginosa* to initiate infection and to persist in a host.

This present study clearly advances our knowledge of potential molecular interactions at the *P. aeruginosa* host/pathogen interface by demonstrating for the first time that the two structurally diverse QSSMs, 3-oxo-C12-HSL and PQS, can exert differential modulatory effects on pure human T cells:

- Both QSSMs inhibit pure human T cell proliferation with PQS being the more potent anti-proliferative.

- 3-oxo-C12-HSL inhibits IL-2 release while PQS significantly enhanced the production of IL-2 while suppressing pure T cell proliferation.

- Both QSSMs inhibited the release of IFNγ in mouse splenocytes

- 3-oxo-C12-HSL inhibited the release of IL-4 though not as potent as inhibiting the release of IFNγ, while PQS had no significant effect on the production of IL-4.

- 3-oxo-C12-HSL inhibits TNFα release while PQS significantly enhanced the release of this cytokine.

CsA, FK506 and rapamycin have found widespread use in the clinic as immunosuppressive drugs. Their potential in treating immune disorders other than

graft rejection, however, is limited in large by their side effects including nephrotoxicity and neurotoxicity (Hong and Kahan, 2000).   Despite recent developments of new immunosuppressants, there remains an unmet need for less toxic and more widely applicable immunosuppressive agents.   While it in inappropriate to speculate the full clinical significance of these findings until bioavailability can be determined, the innate bioactivity of these chemically defined QSSMs is clear; these activities may yet be harnessed to treat immunological diseases, in the same way present immunosuppressive drugs have been exploited.

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Appendix 1 – Buffers and Reagent Properties

A.1.1. Cell Culture

A.1.1.2. Complete medium

RPMI 1640	475 ml
Fetal calf serum (FCS) heat inactivated	25 ml
L-glutamine (200 mM)	5 ml
Penicillin/Streptomycin (10,000 units/ml : 10mg/ml)	5 ml
2-ME	2 µl

A.1.1.3. 0.5 M EDTA

EDTA	84.05 g
dH$_2$O	500 ml

A.1.1.4. Red cell lysis buffer (pH 7.3)

Ammonium chloride	144 mM
Tris base	17 mM

Titrate with concentrated HCl to pH 7.2 and filter sterilise

A.1.2. T cell isolation

A.1.2.2. 2 M EDTA stock solution

EDTA	0.684 mg
dH$_2$O	100 ml

A.1.2.3. T cell isolation buffer

PBS	100 ml
BSA	0.5 g
2 M EDTA	0.1 ml

A.1.3. FACs analysis

A.1.3.1. Phosphate buffered saline (PBS) solution (10 L)

Sodium chloride	80 g
Dibasic sodium phosphate	11.6 g
Potassium dihydrogen phosphate	2 g
Potassium chloride (KCl)	2 g

Adjust to pH 7.0 and make up to 10 L with dH_2O

A.1.3.2. PBA

BSA	30 g
0.02% sodium azide	0.02 g

Adjust to 100 ml with PBS

A.1.4. ELISA

A.1.4.1. Binding solution

Dibasic sodium phosphate	35.49 g
dH_2O	500ml

Adjusted to pH 9 with 0.1 M sodium dihydrogen phosphate

A.1.4.2. 0.1 M Sodium dihydrogen phosphate

Sodium dihydrogen phosphate	34.50 g
dH_2O	500 ml

A.1.4.3. PBS/Tween-20

Tween-20	0.5 ml
PBS	1 L

A.1.4.4. Blocking buffer

1% BSA

PBS

A.1.4.5. Blocking buffer/Tween-20

Tween-20	0.5 ml
Blocking buffer	1 L

A.1.4.6. 0.05 M Phosphate-citrate buffer (pH 5)

Solution I:

Dibasic sodium phosphate	28.39 g
dH$_2$O	1 L

Solution II:

Citric acid monohydrate	10.51 g
Sodium phosphate	14.20 g
Adjust to pH 5 with solution I	
Adjust to 1 L with dH$_2$O	

A.1.4.7. TMB solution

3,3',5,5,'-tetramthylbenzidine (TMB)	1 Tablet
DMSO	1 ml
0.05 M Phosphate-citrated buffer (pH 5)	9 ml
30% Hydrogen peroxide	2 µl

A.1.4.8. 0.05 M Phosphate-citrate buffer (pH 5)

Phosphate-citrate buffer	1 Tablet
dH$_2$O	100ml

A.1.4.9. 30% Hydrogen peroxide

Hydrogen peroxide	30 ml
dH$_2$O	100 ml

A.1.5. SDS-PAGE (Sodium dodecyl sulphate – polyacrylamide gel electrophoresis)

A.1.5.1. 12% Resolving gel

Acrylamide/bis-acrylamide (29.2% : 0.8% w/v)	4.8 ml
1.5 M Tris-HCL (pH 8.8)	3 ml
10% SDS (Sodium dodecyl sulphate)	120 μl
dH$_2$O (Distilled water)	4.08 ml
Ammonium persulphate (10%)	60 μl
TEMED (N,N,N'mN'-tetramethylene-ethylenediamine)	6 μl

A.1.5.2. 4% Stacking gel

Acrylamide/bis-acrylamide (29.2% : 0.8% w/v)	660 μl
0.5 M Tris-HCL (pH 6.5)	1.25 ml
10% SDS	50 μl
dH$_2$O	3.04 ml
Ammonium persulphate (10%)	50 μl
TEMED (N,N,N'mN'-tetramethylene-ethylenediamine)	20 μl

A.1.5.3. Reducing sample buffer (10ml)

1 M Tris-HCL (pH 6.8)	0.6 ml
50% Glycerol	5 ml
10% SDS	2 ml

2-ME	0.5 ml
1% Bromophenol blue	1 ml
dH$_2$O	0.9 ml

A.1.5.4. Running buffer (10 L)

Tris base	30 g
Glycine	144 g
SDS	10 g
Make up to 10 L with dH$_2$O	

A.1.5.5. Transfer buffer (1 L)

Tris base	3.63 g
Glycine	14.4 g
20 % Methanol (*v/v*)	200 ml
0.038 % SDS	0.37 g
Make up to 1 L with dH$_2$O	

A.1.6. Western Blotting

A.1.6.1. 1 x TBS (1 L)

NaCl	87.66 g
Tris	12.11 g
pH 8.0	
Make up to 1L with dH$_2$O	

A.1.6.2. TBS/0.05% Tween-20

| 1 x TBS | 500 ml |
| Tween-20 | 250 μl |

A.1.6.3. Blocking Buffer

Bovine serum albumin (BSA)	0.3 g
1 x TBS	30 ml

A.1.6.4. Primary and Secondary antibody buffer

BSA	0.03 g
1 x TBS	30 ml

A.1.6.5. 0.75M Tris pH 9.6

Tris base	45.41 g
Make up to 500ml with dH_2O	

A.1.6.6. Alkaline phosphatase substrate

5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in PMF)	33 μl
Nitro blue tetrazolium (70 mg/ml) in 70% PMF)	44 μl
Made up to 20 ml 0.75 M Tris pH 9.6	

A.1.7. Isolation of total RNA

A.1.7.1. 70% Ethanol (100ml)

Ethanol	70 ml
RNase-free water	30 ml

A.1.8. RNA isolation and dye incorporation

A.1.8.1. Reverse transcription master mix (for 1 sample)

10x First strand buffer	2 μl
dNTP mix	4 μl

| RNase inhibitor | 1 μl |
| ArrayScript | 1 μl |

A.1.8.2. Second strand master mix (for 1 sample)

Nuclease free water	63 μl
10x Second strand buffer	10 μl
dNTP mix	4 μl
DNA polymerase	2 μl
RNase H	1 μl

A.1.8.3. IVT master mix for 2 rounds of amplification (for 1 sample)

ATP, CTP, GTP mix (50mM)	12 μl
UTP solution (50mM)	6 μl
T7 10x reaction buffer	4 μl
T7 enzyme mix	4 μl

A.1.8.4. Second strand master mix for second round amplification

Nuclease free water	58 μl
10x second strand buffer	10 μl
dNTP mix	4 μl
DNA polymerase	2 μl

Appendix 2 – Results of individual experiments

A.2.1. Stimulatory effect of ConA on murine splenocytes (BALB/c mice). 1 µg/ml ConA gives optimal cell proliferation.
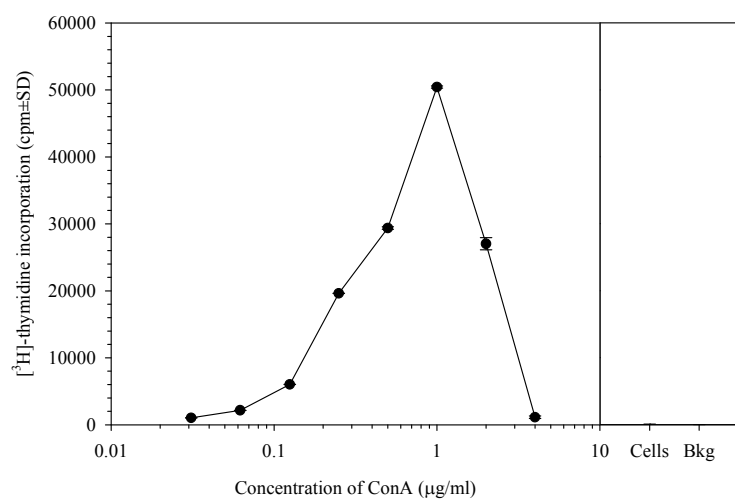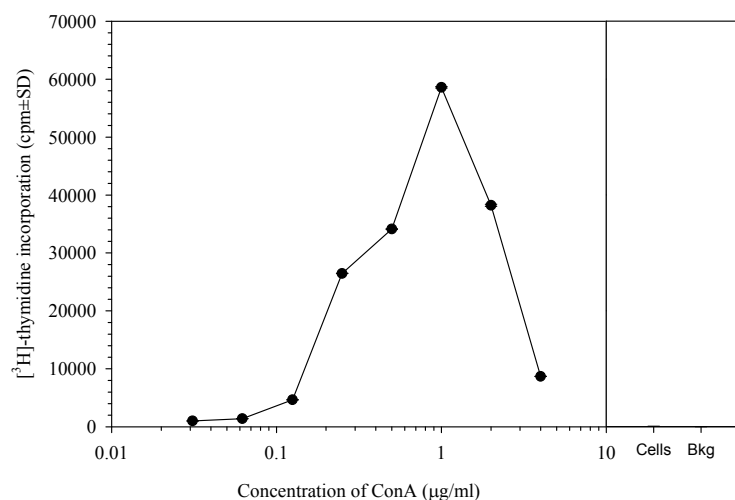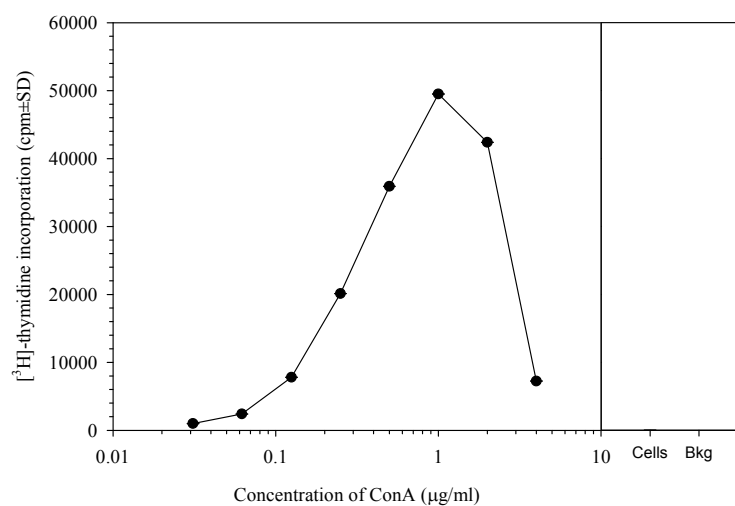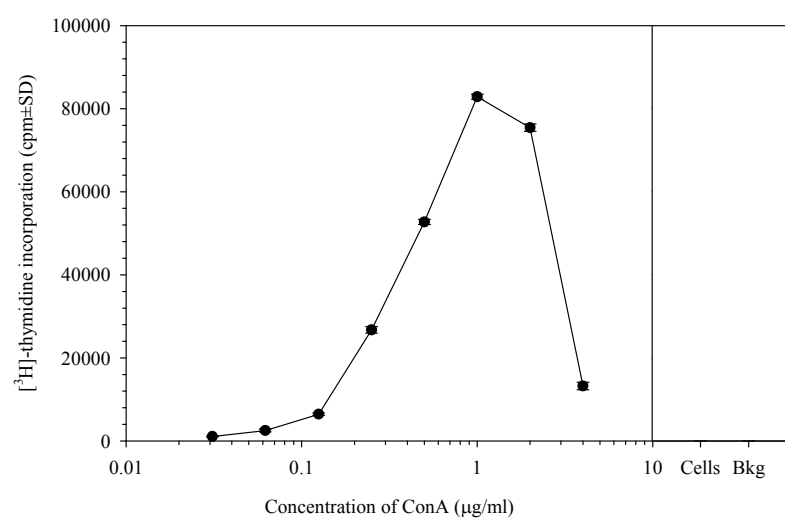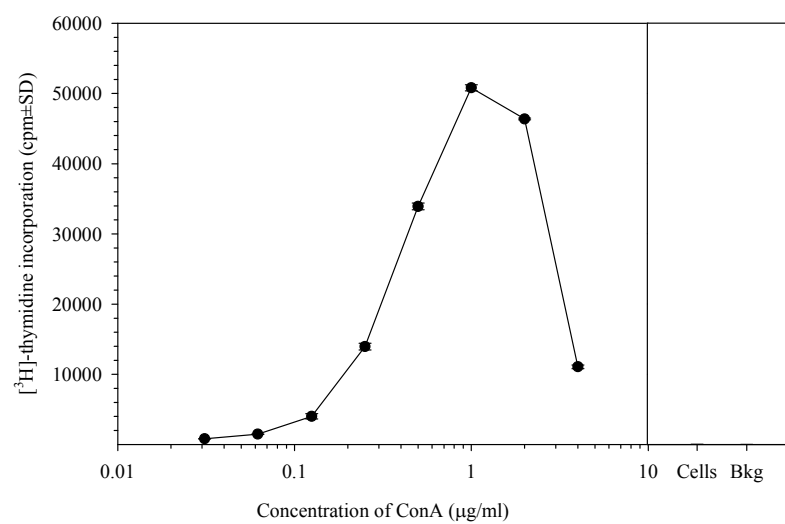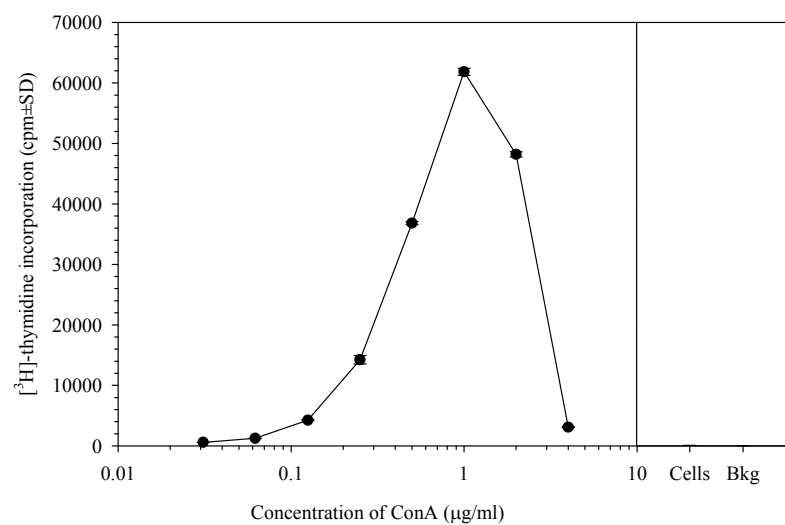
A.2.2. Stimulatory effect of anti-CD3 on murine splenocytes (BALB/c mice).

3 µg/ml anti-CD3 gives optimal cell proliferation.
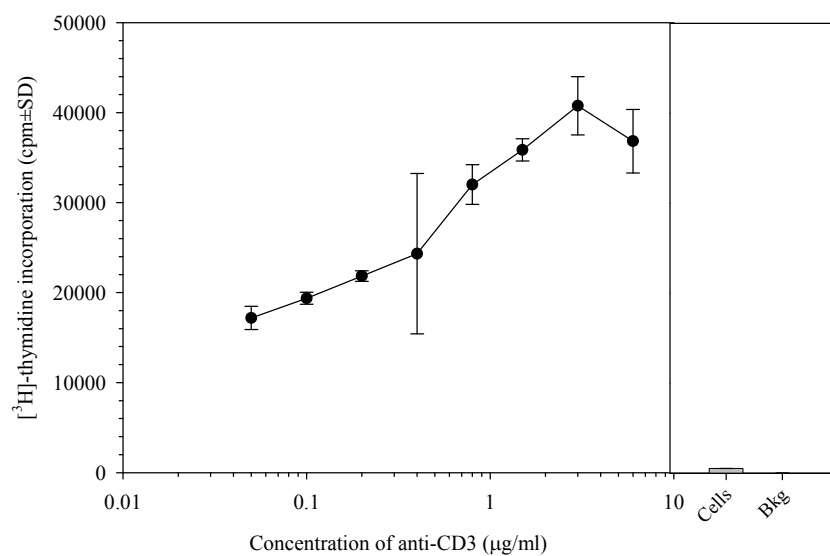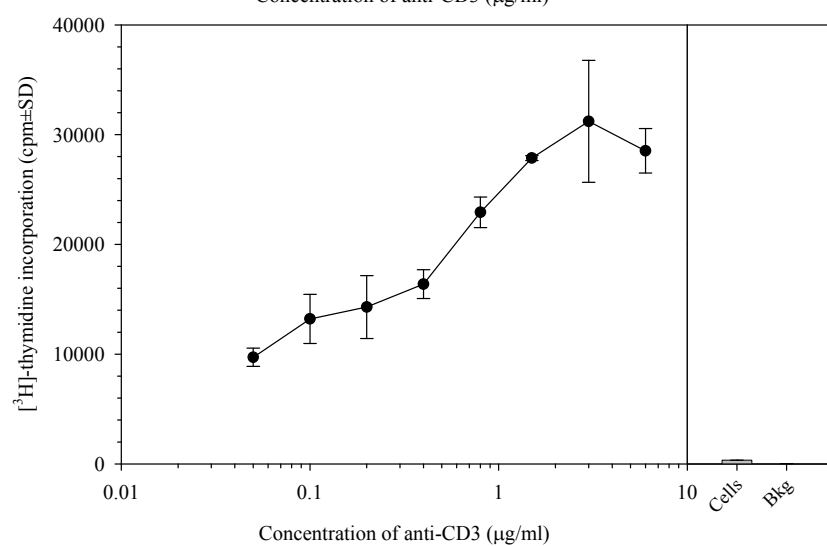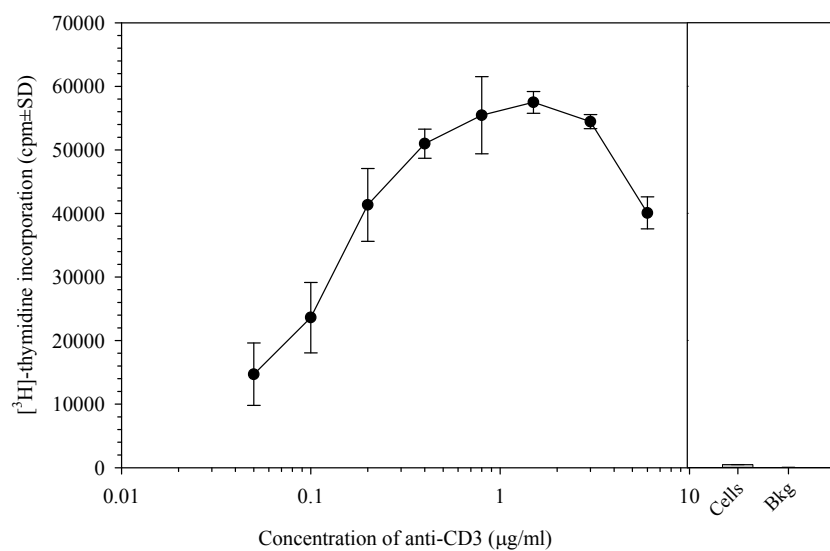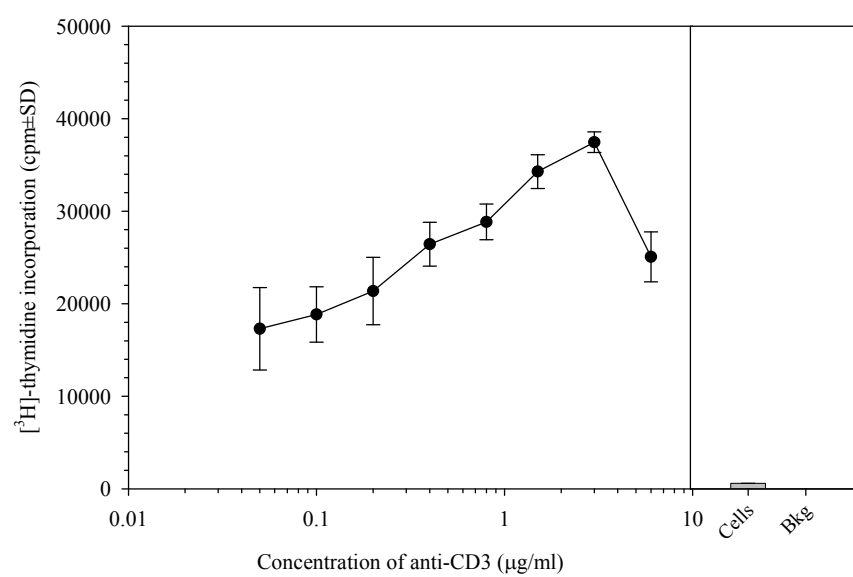
A.2.3. The effect of *P. aeruginosa* QSSMs and CsA on ConA driven splenocyte proliferation. ***P<0.001, **P<0.01, *P<0.05 compared with the ConA control. Cells represent untreated cells and Bkg (background) represent readings for CTCM.
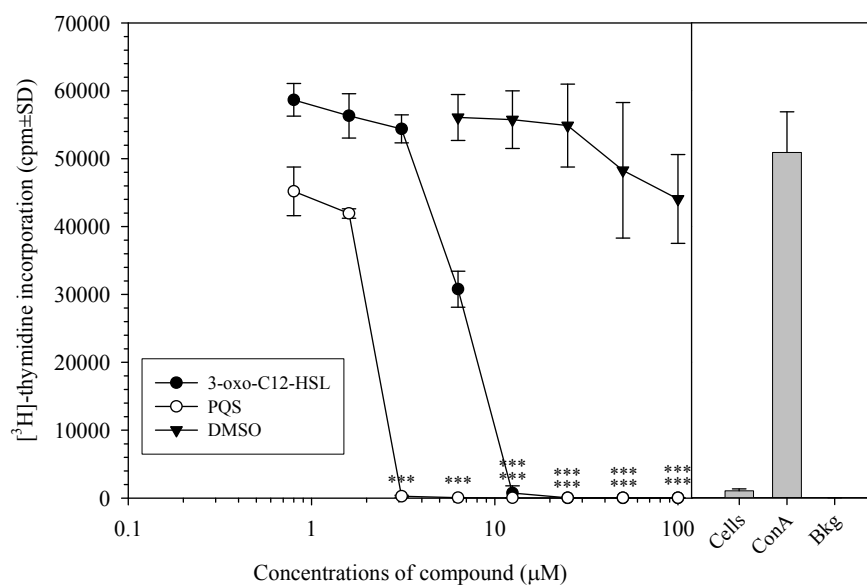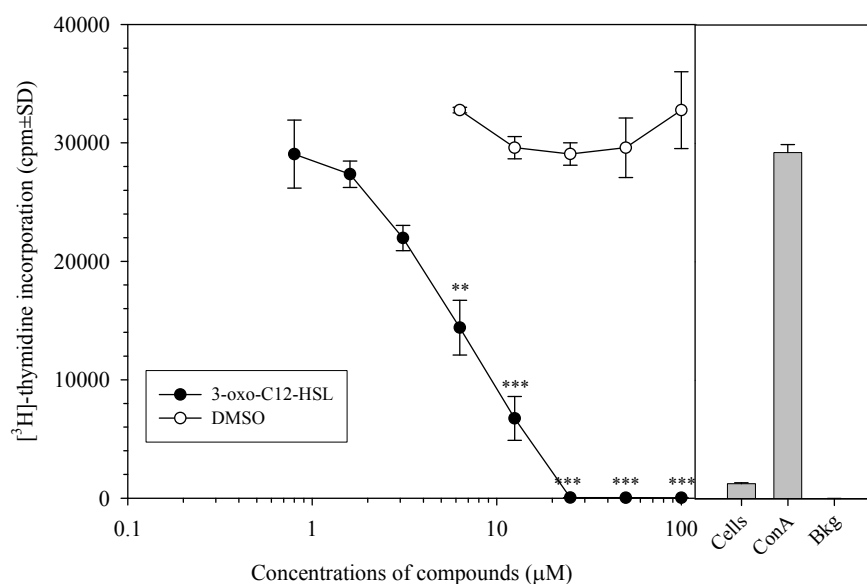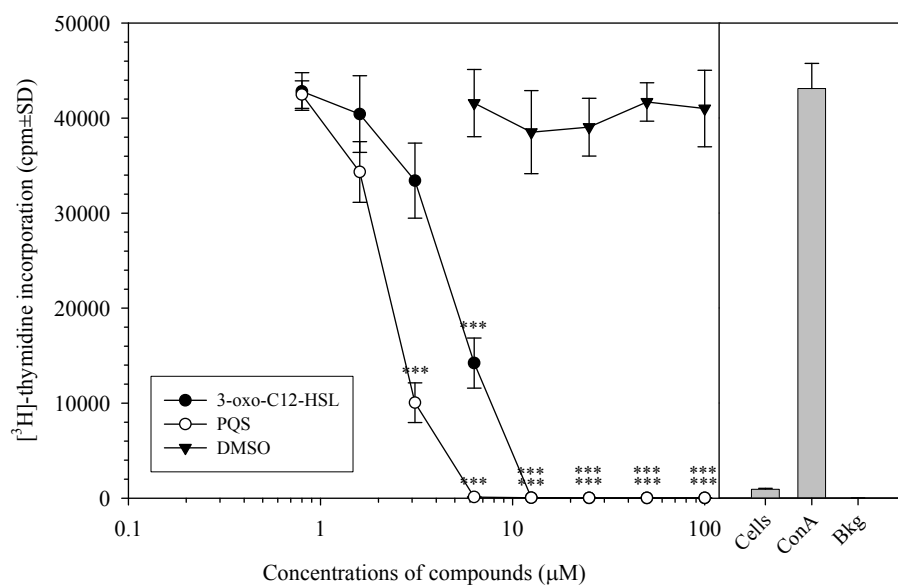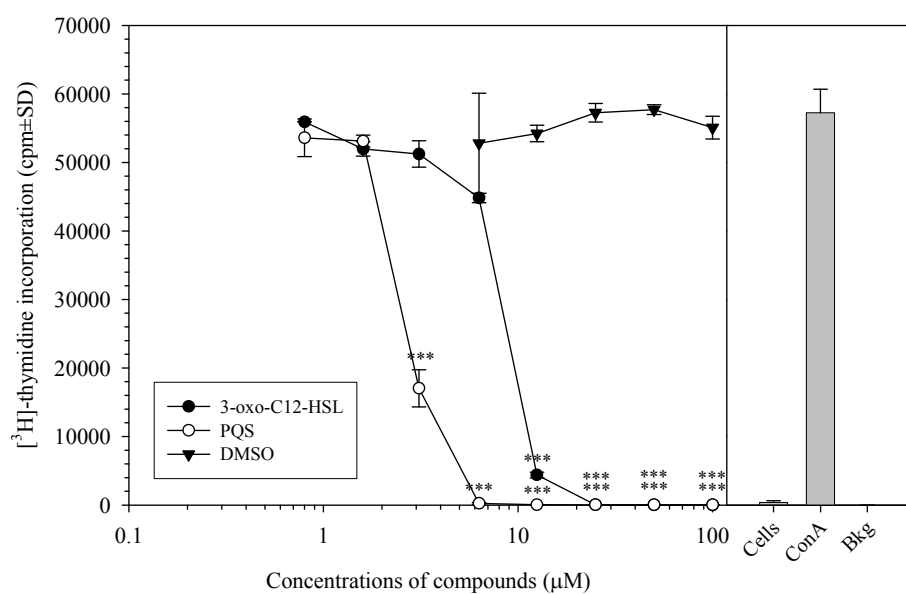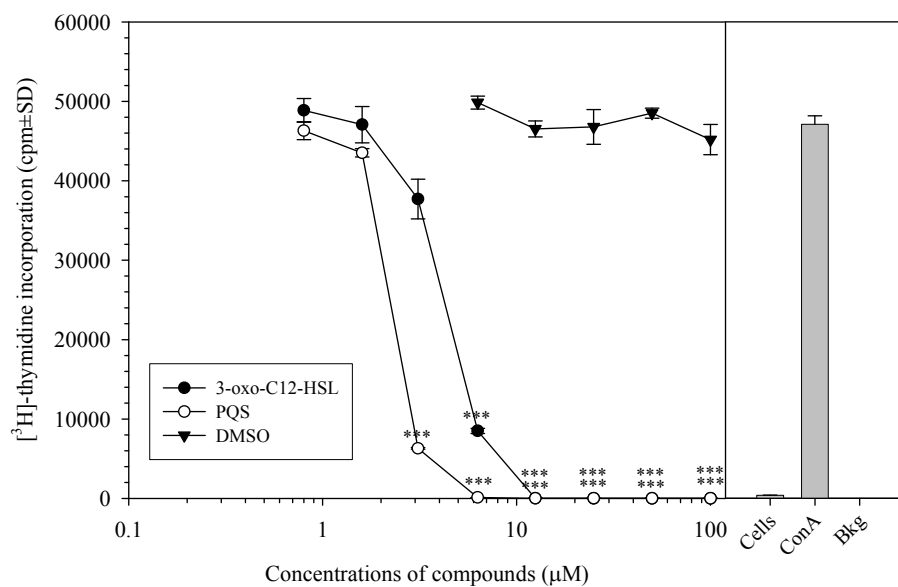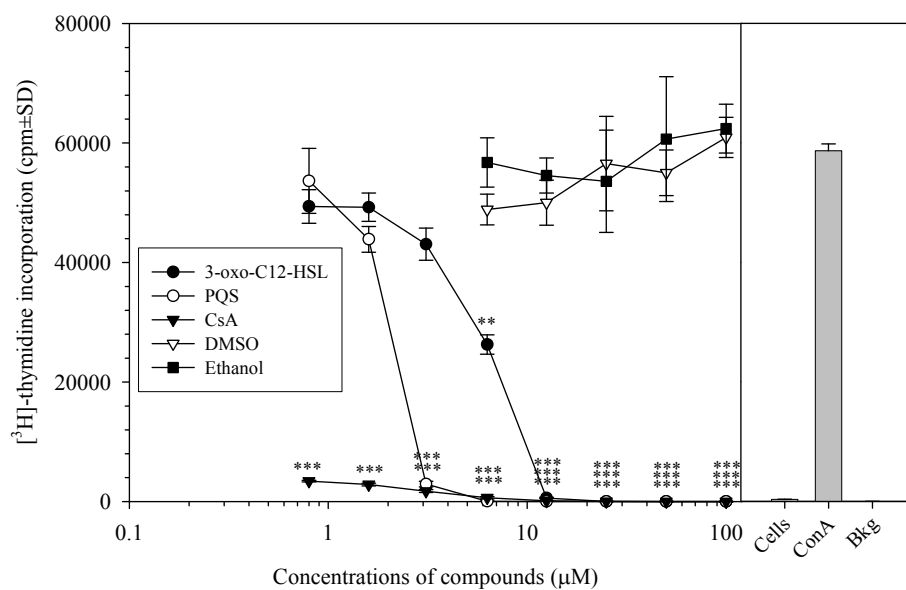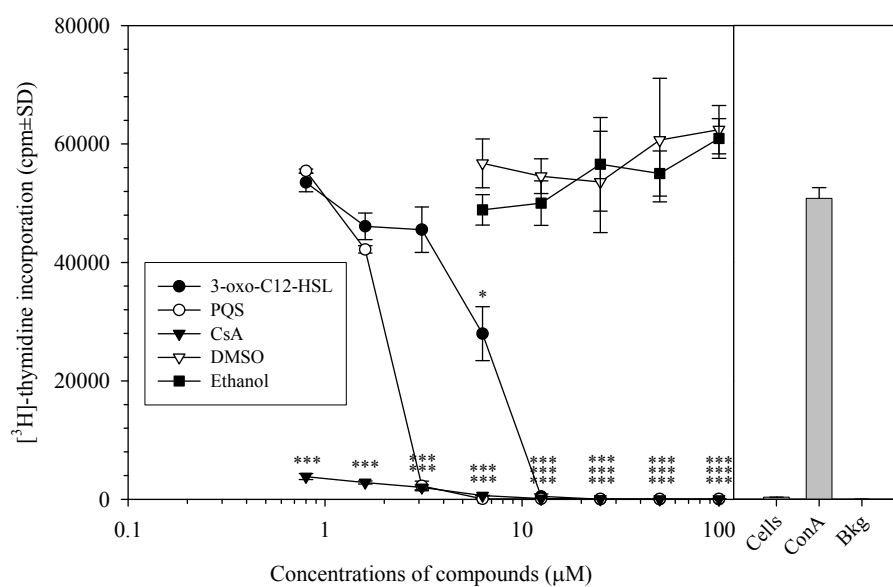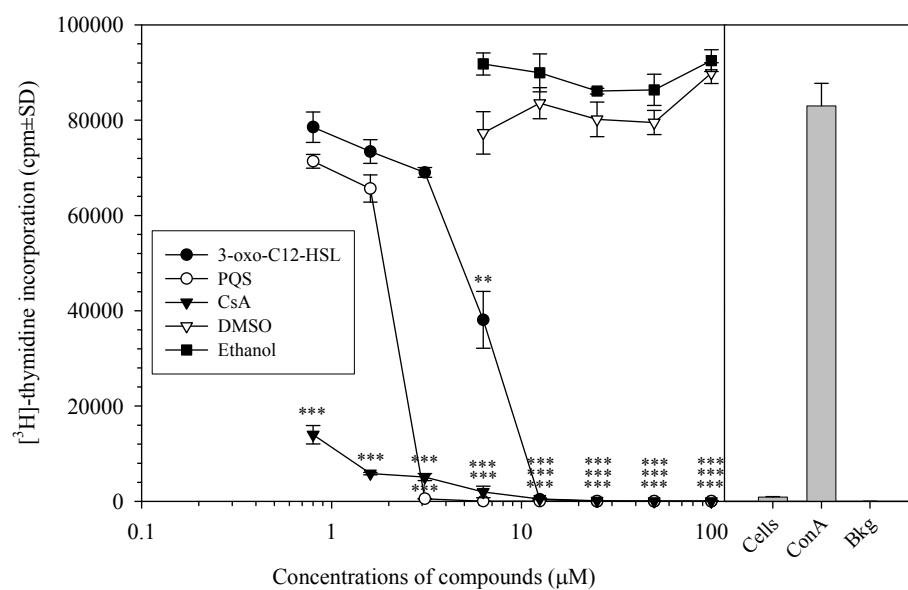
A.2.4. **The effect of *P. aeruginosa* QSSMs and CsA on anti-CD3 driven splenocyte proliferation.** ***P<0.001, **P<0.01, *P<0.05 compared with the anti-CD3 control. Cells represent untreated cells and Bkg (background) represents measurement for CTCM.
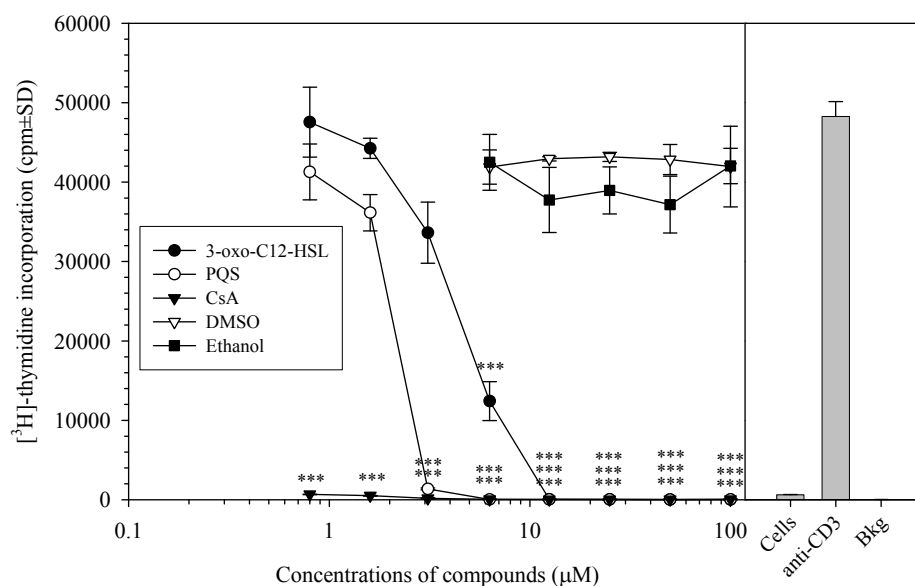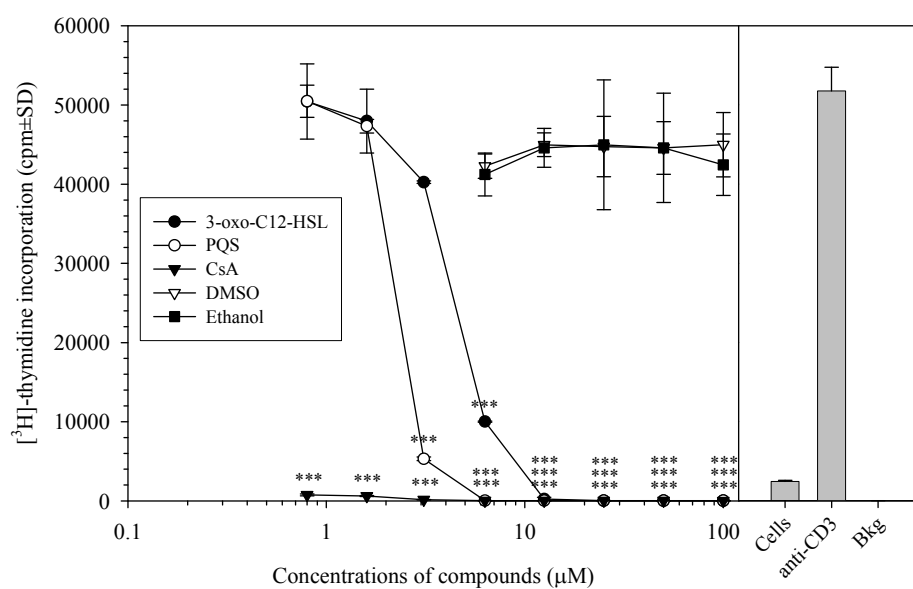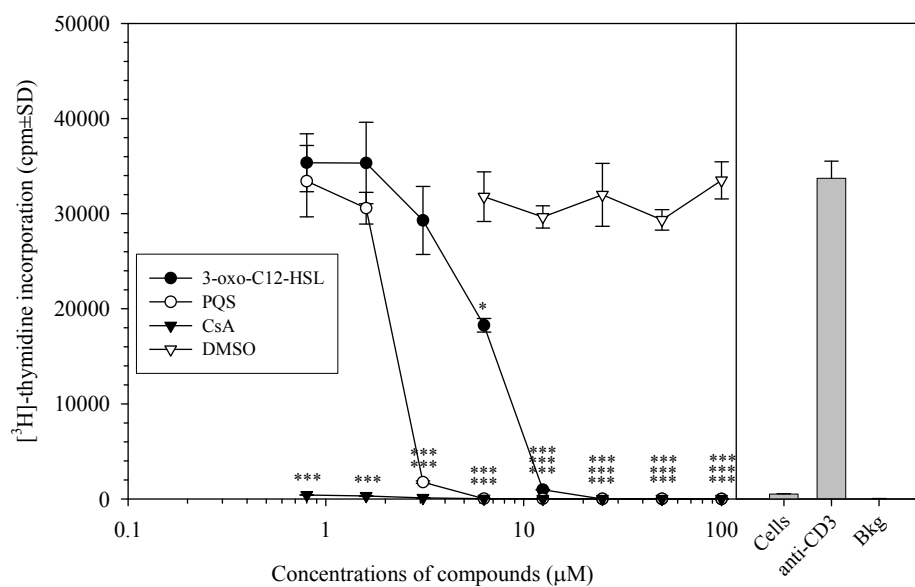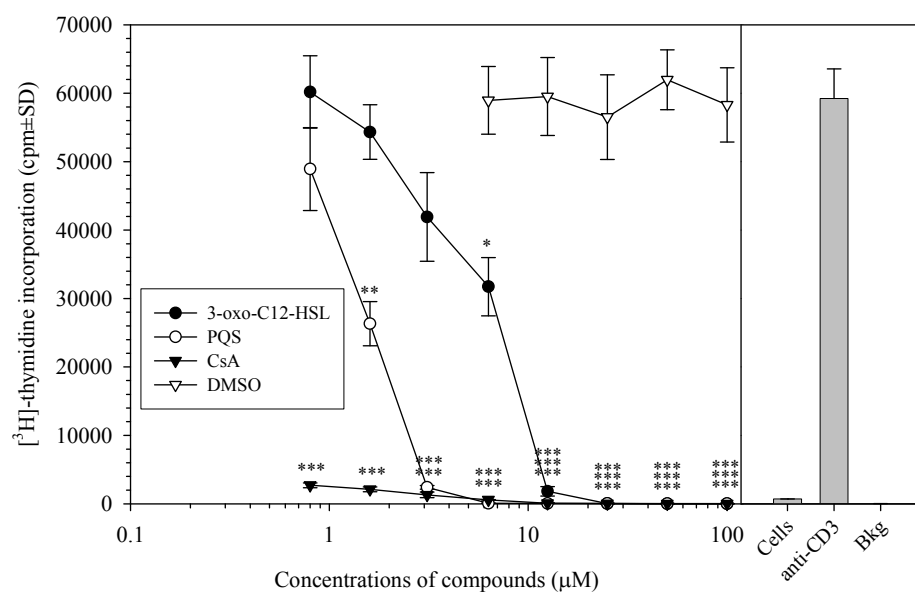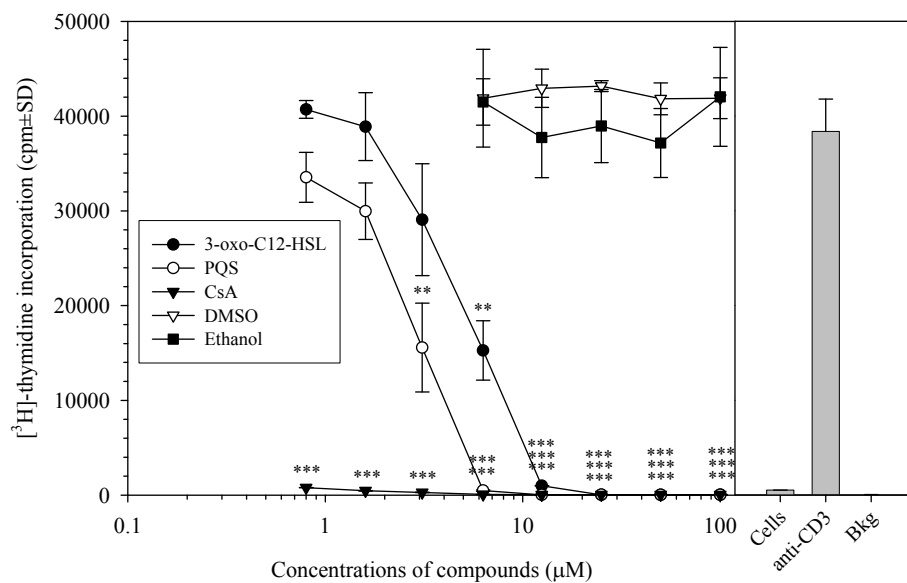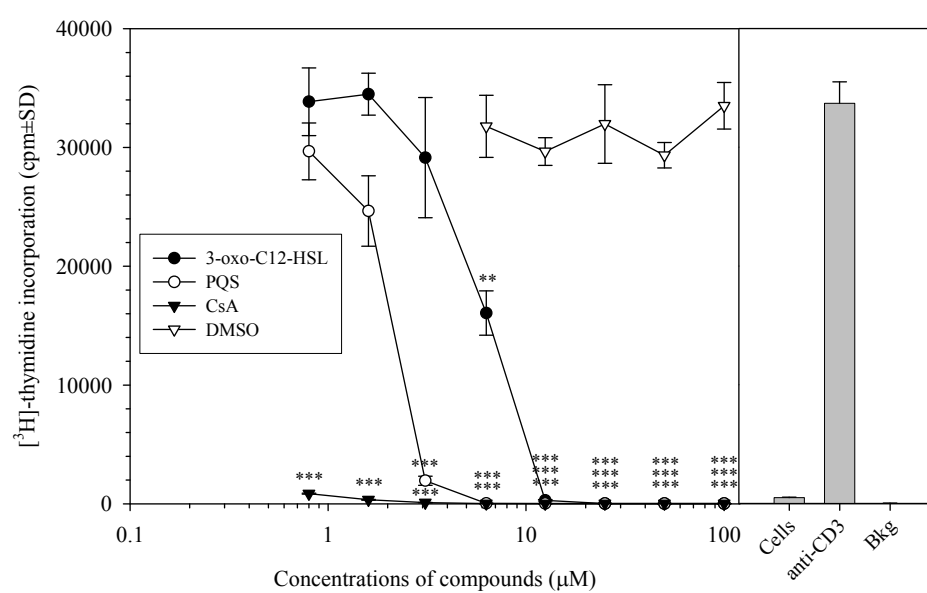
A.2.5. **The effect of *P. aeruginosa* QSSMs and CsA on murine splenocyte viability.** Cell control represents untreated cells and Bkg (background) represents measurement of CTCM.
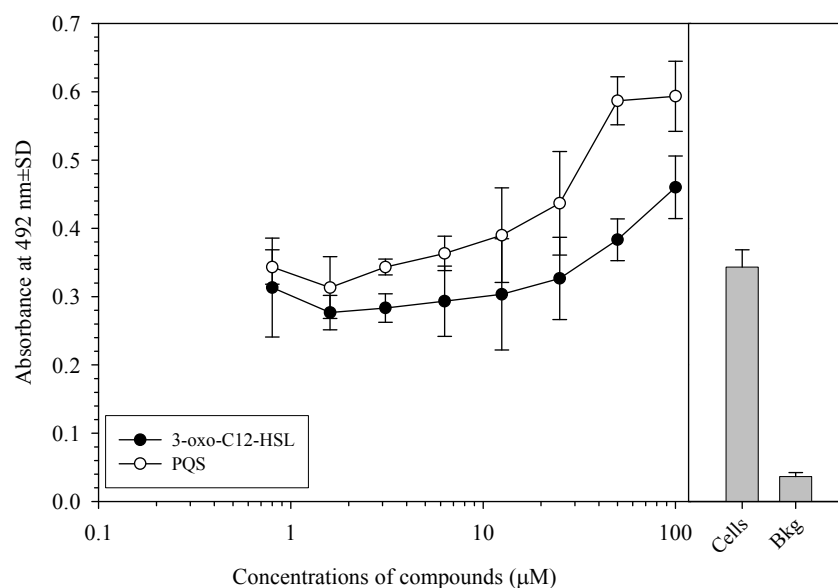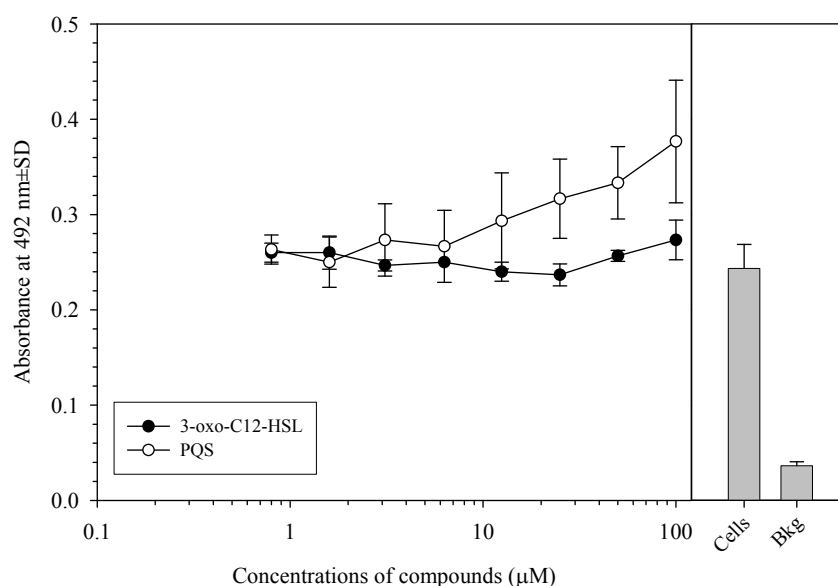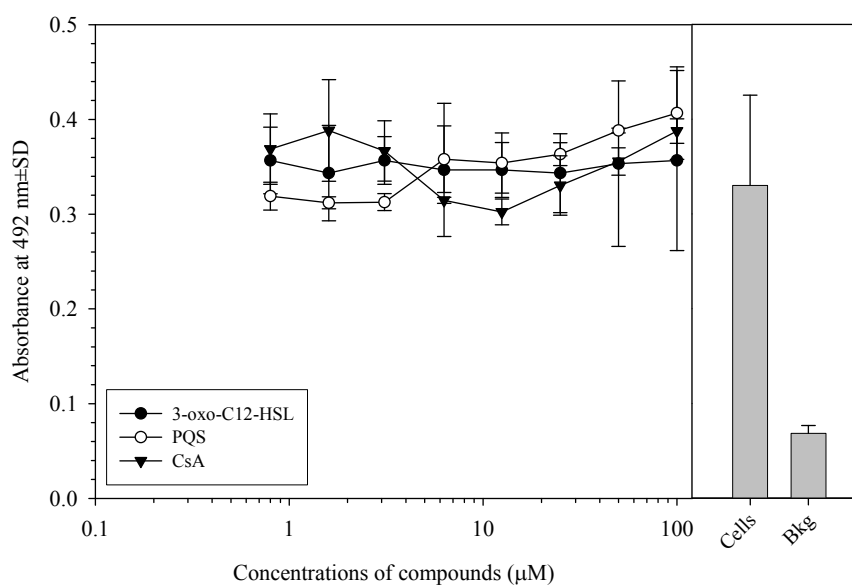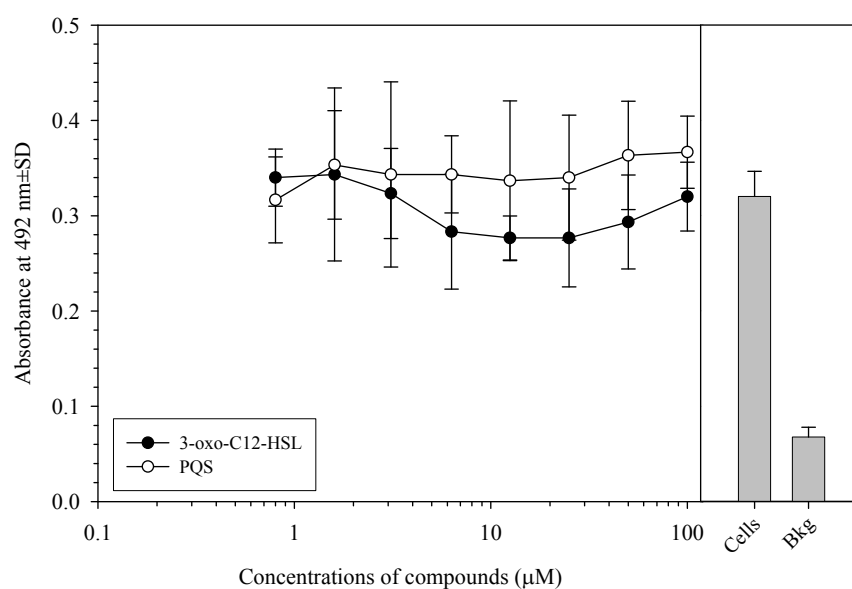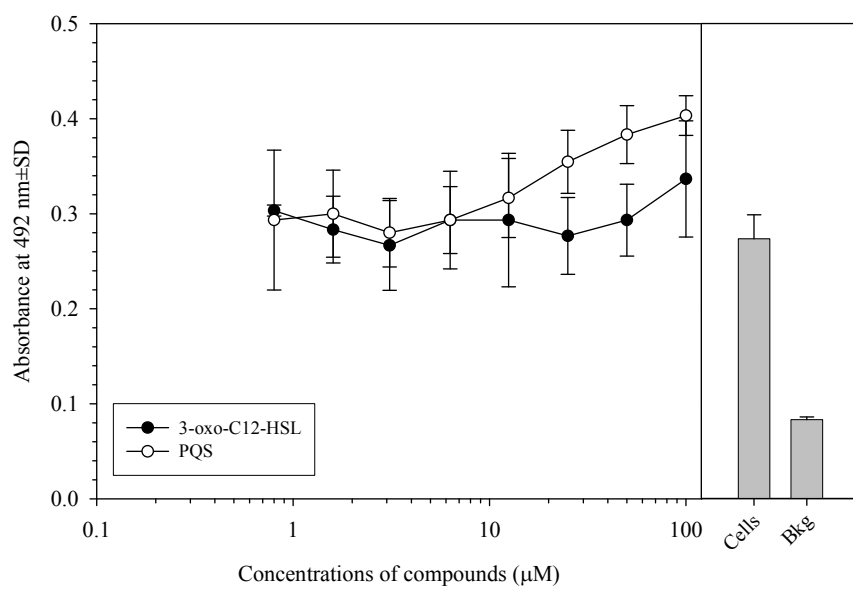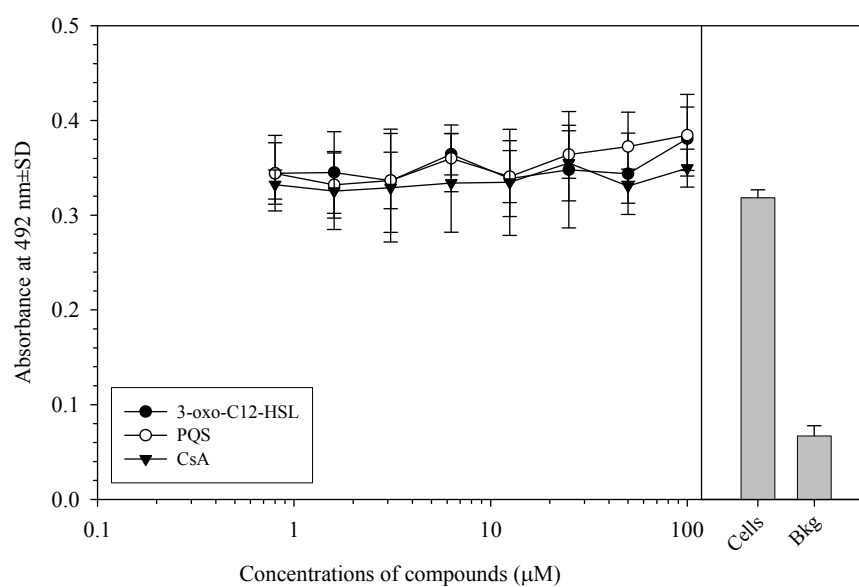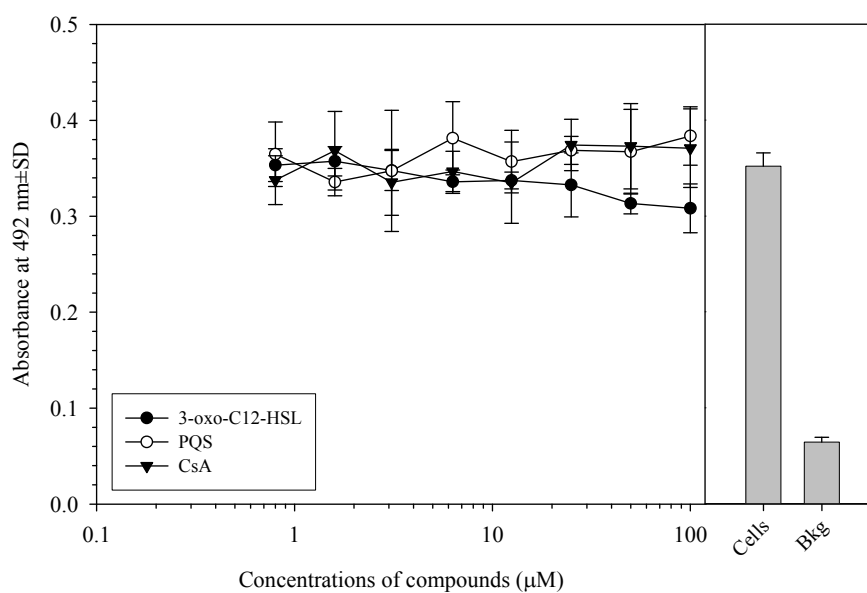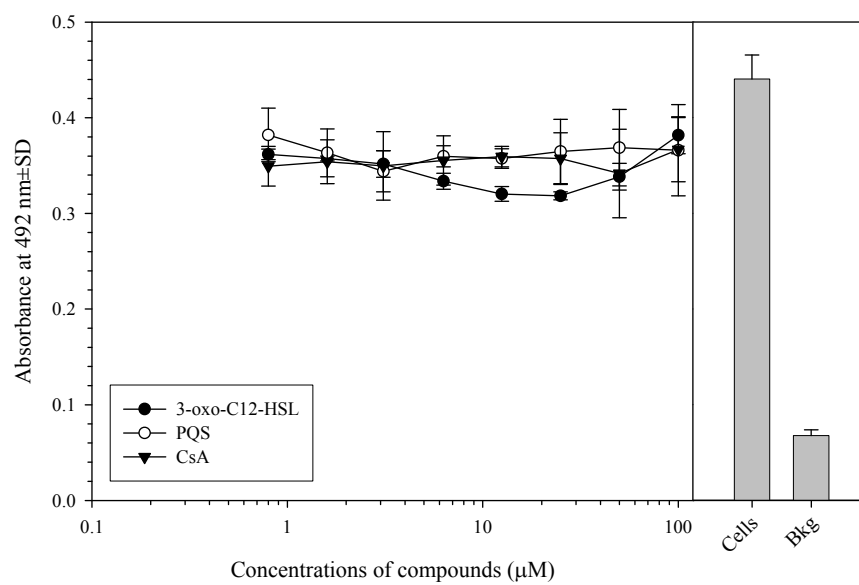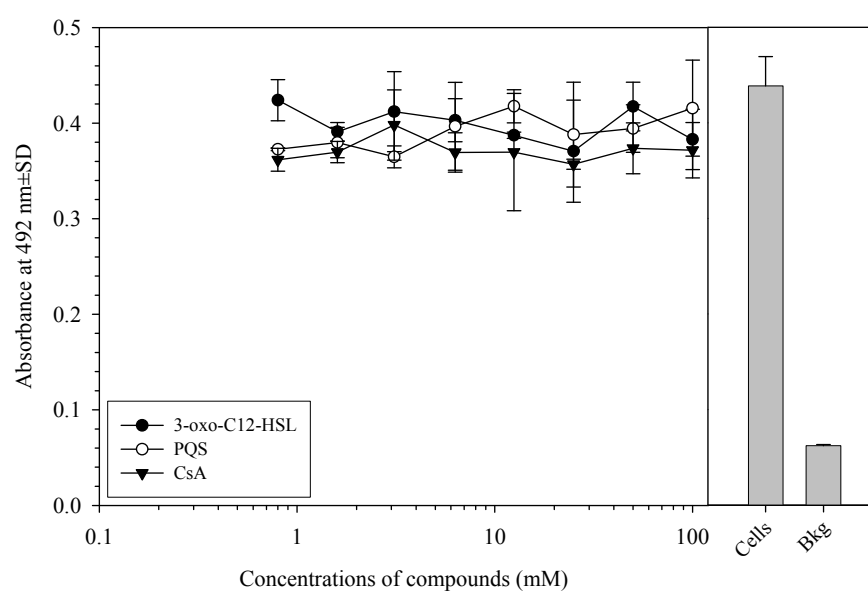
A.2.6. **The dose response of anti-CD3 and anti-CD28 on human PBMC proliferation.** The concentration of anti-CD3 and antiCD28 that gives optima proliferation is 0.1 μg/ml and 5 μg/ml respectively. 1 = 0.003/0.15. 2 = 0.006/0.312, 3 = 0.012/0.62, 4 = 0.025/1.25, 5 = 0.05/2.5, 6= 0.1/5, 7 = 0.2/10, 8 = 0.4/20 μg/ml of anti-CD3 and anti-CD28 respectively.
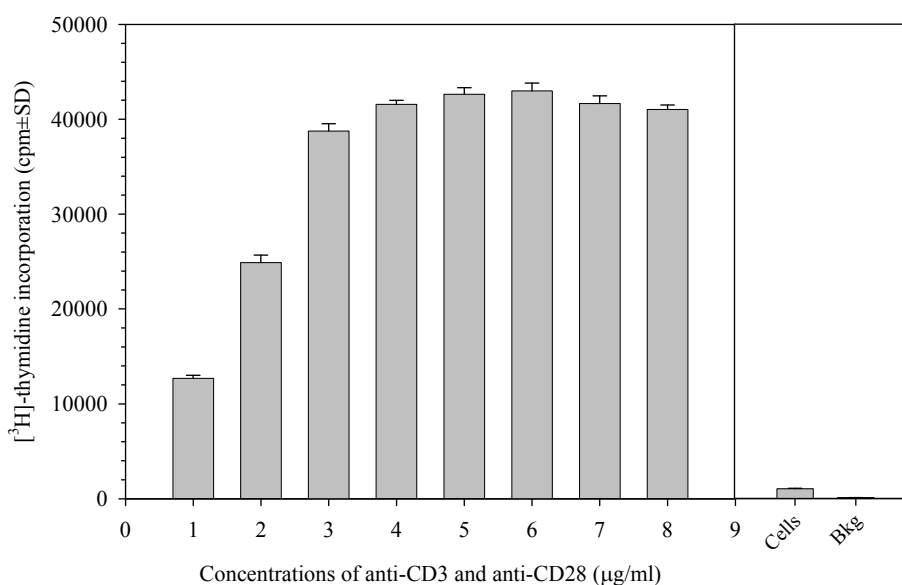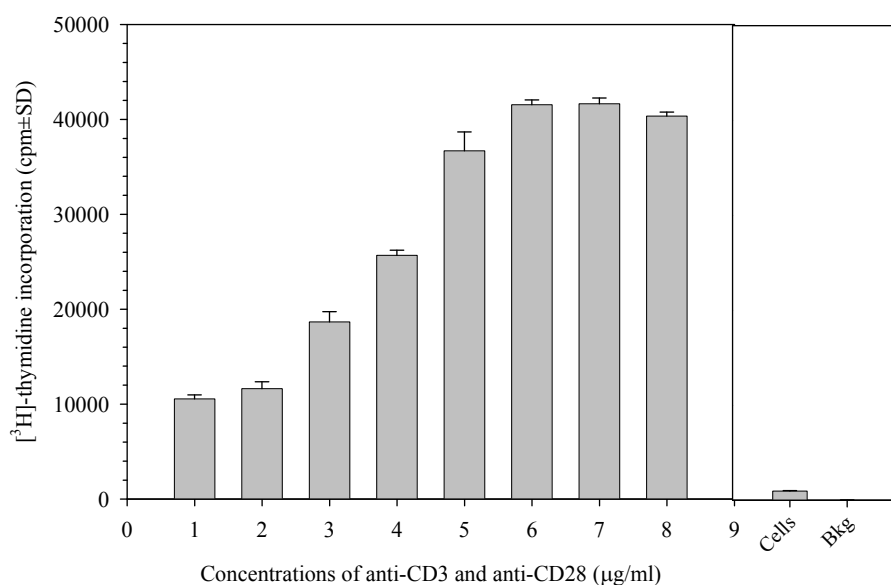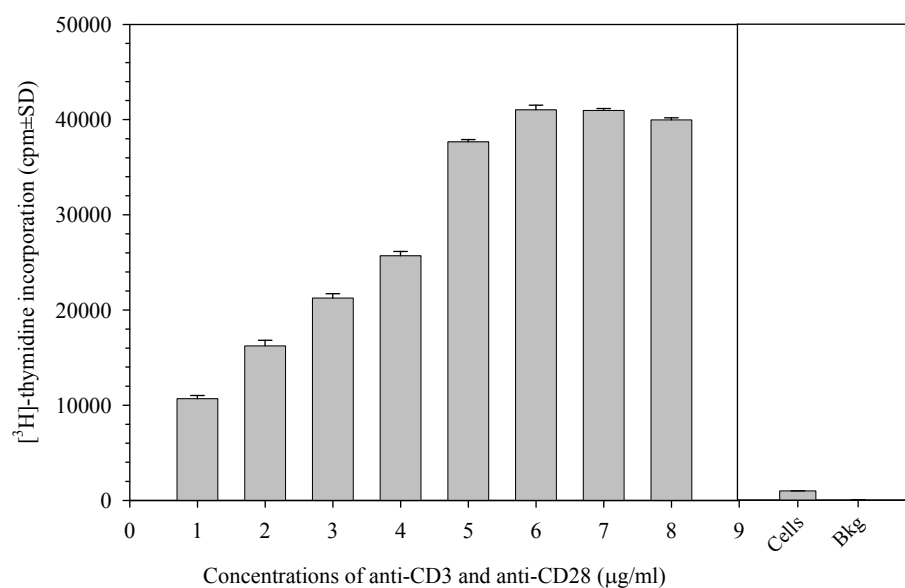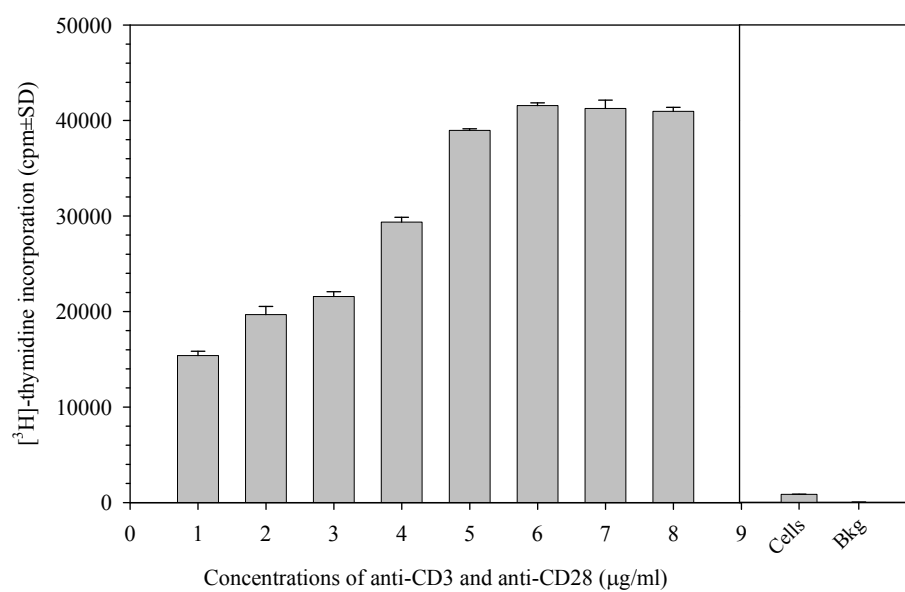
A.2.7. The effect of *P. aeruginosa* QSSMs and CsA on anti-CD3 and anti-CD28 driven human PBMC proliferation. ***P<0.001, **P<0.01, *P<0.05 compared with the anti-CD3 and anti-CD28 control. Cells represent untreated cells and Bkg (background) is measurements for CTCM.
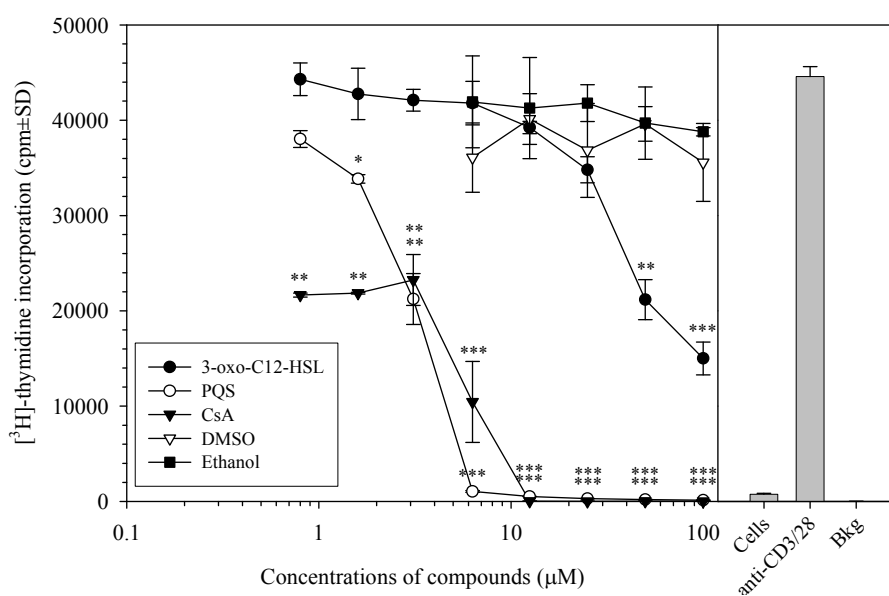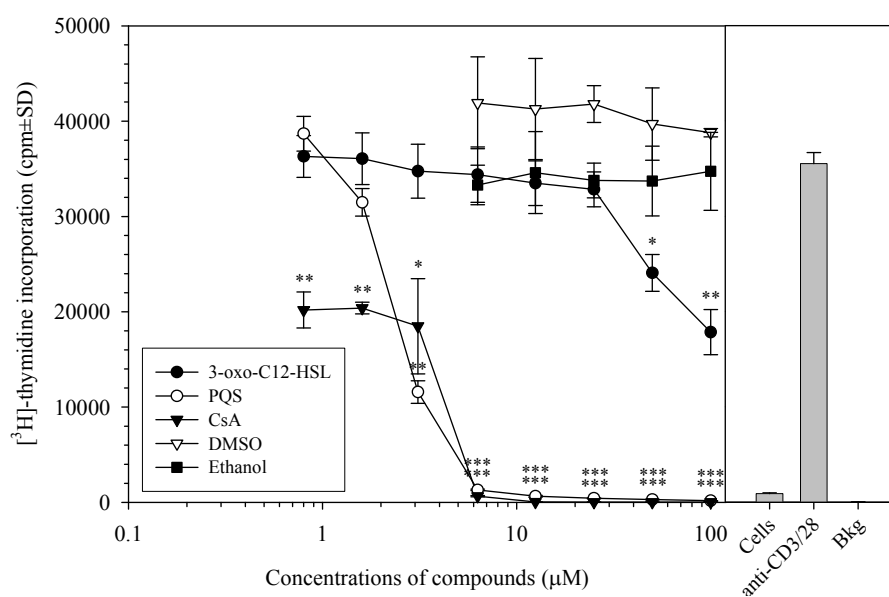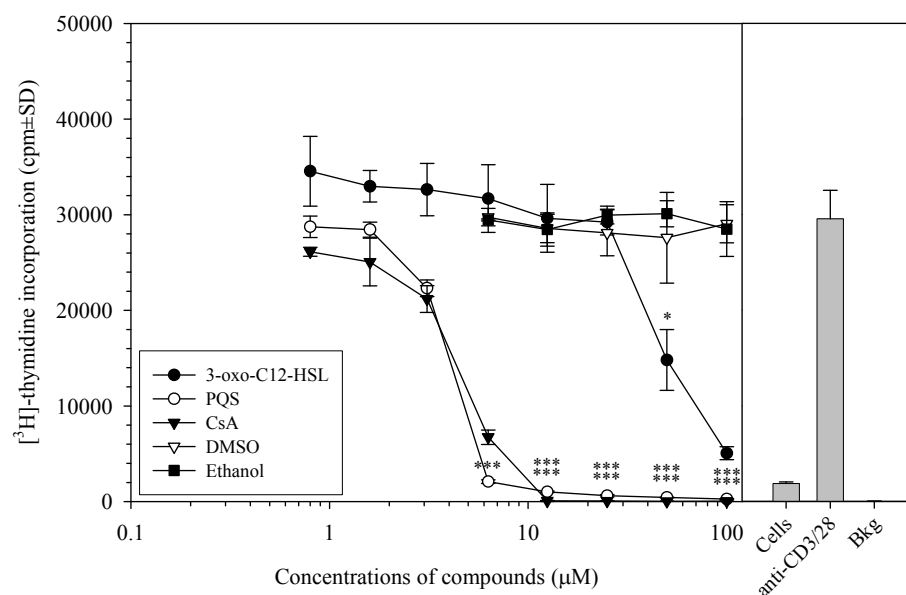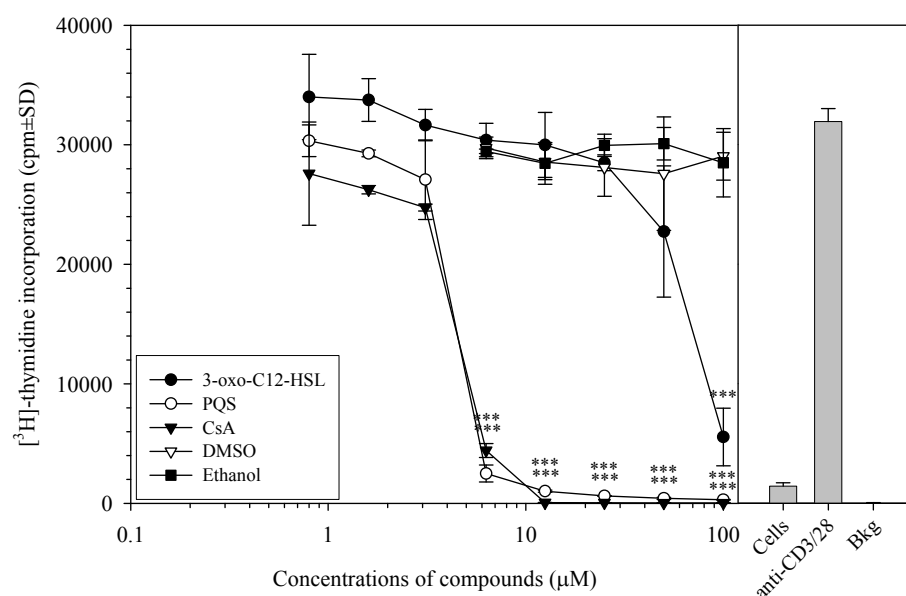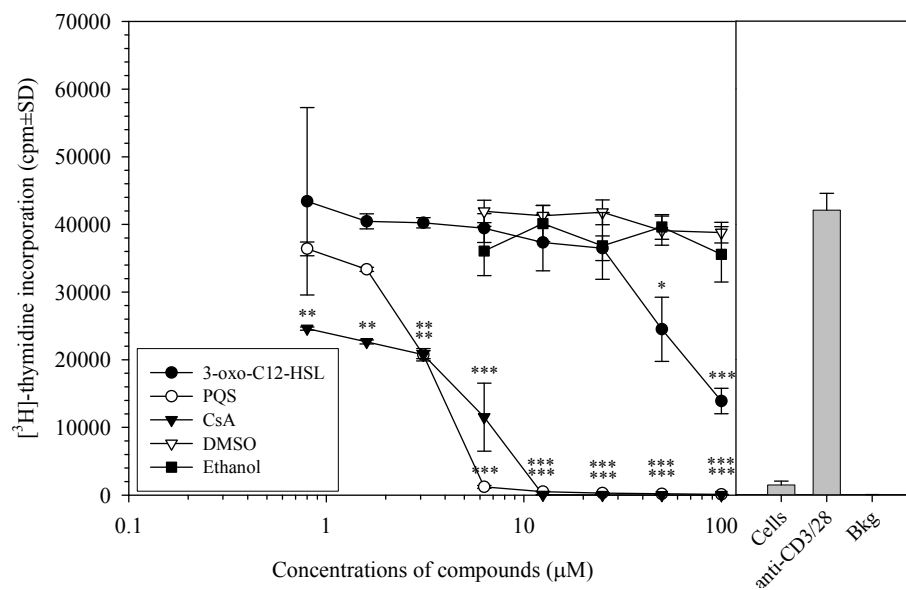
A.2.8. The effect of *P. aeruginosa* QSSMs and CsA on human PBMC viability.

*P<0.05 compared to the cell control (untreated cells). Bkg (background) represents
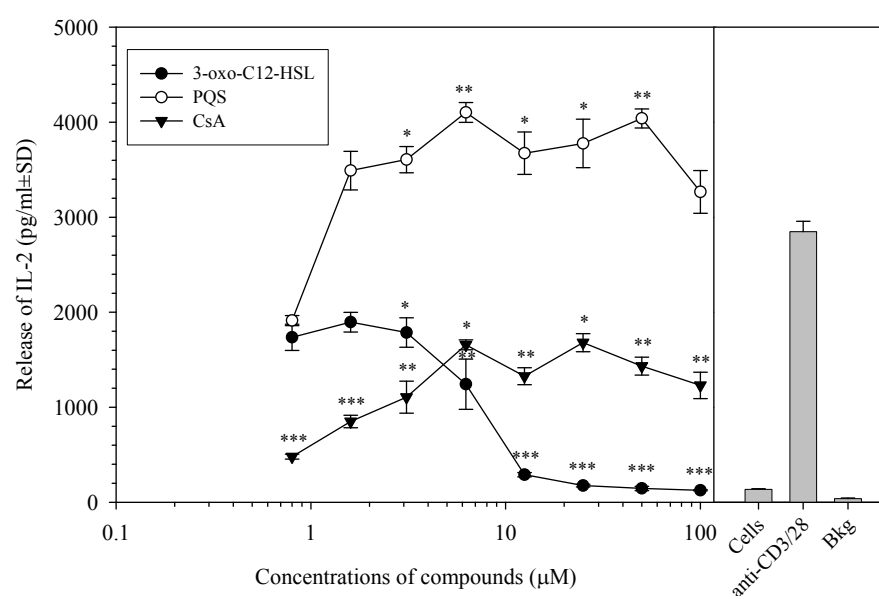
measurement of CTCM.
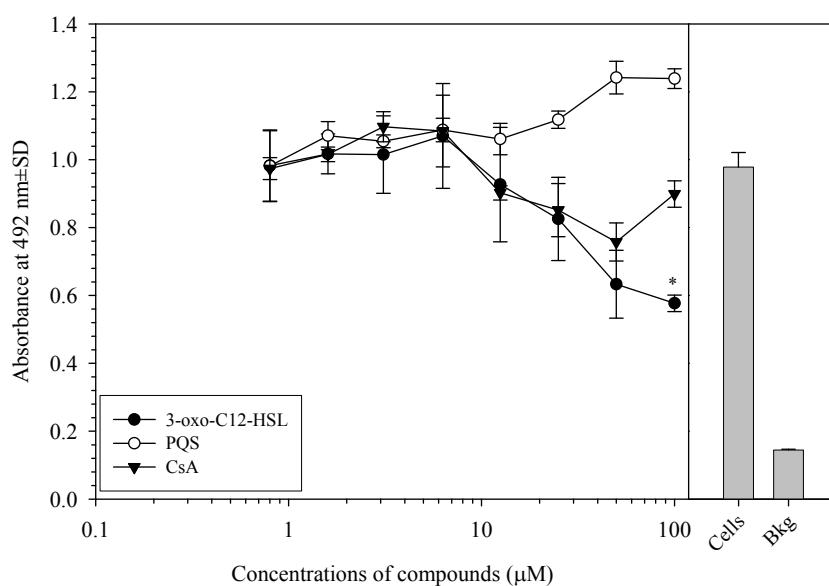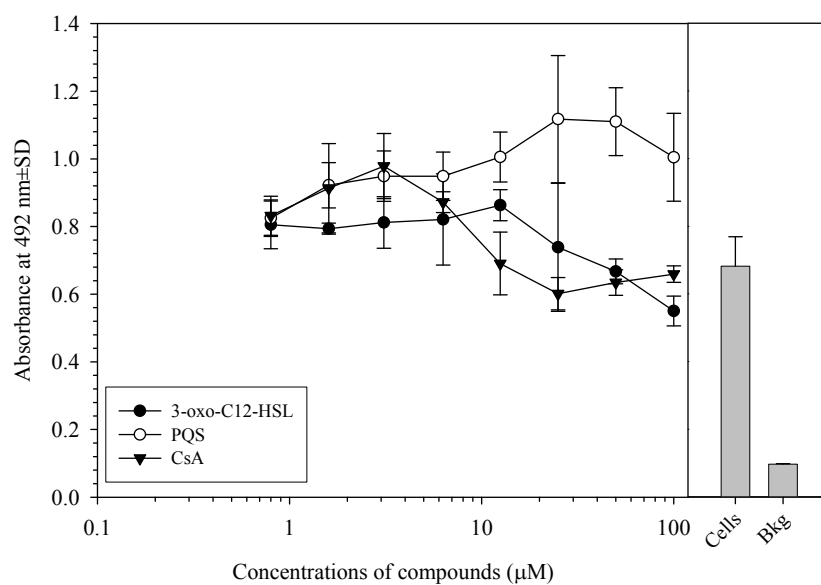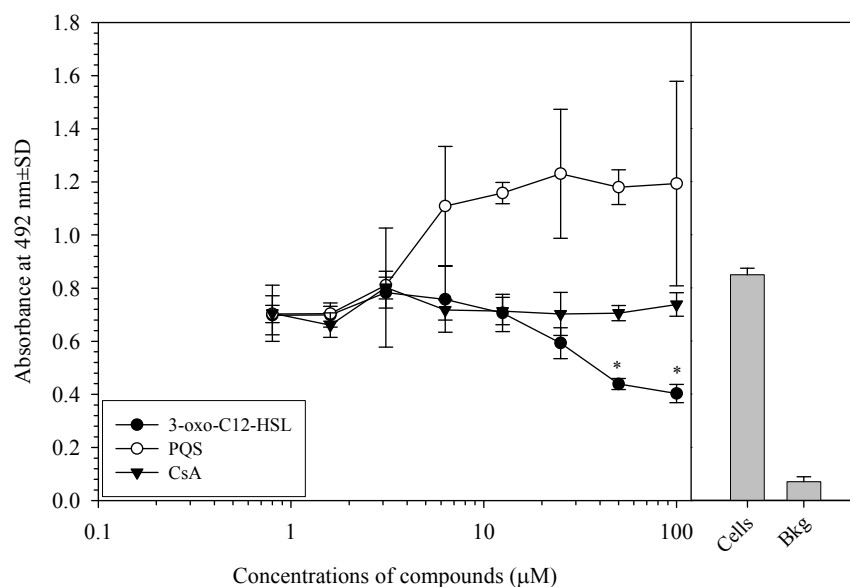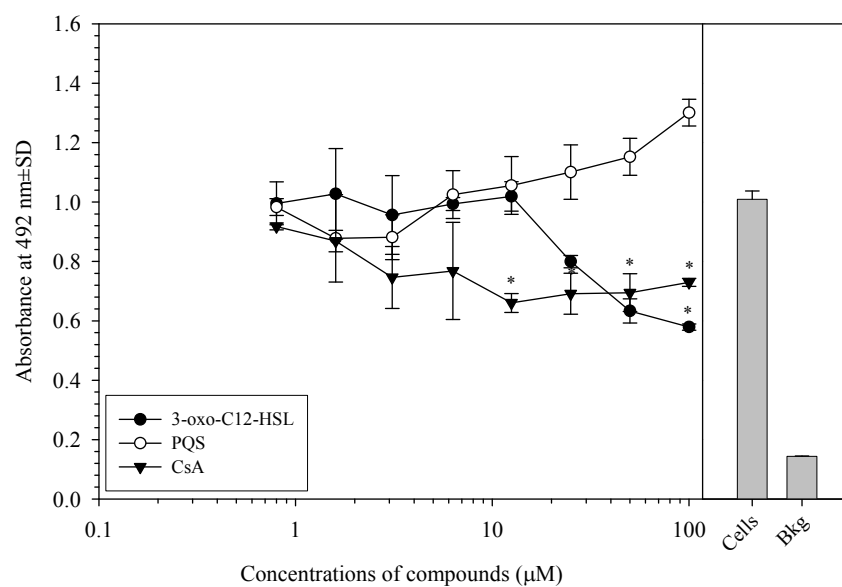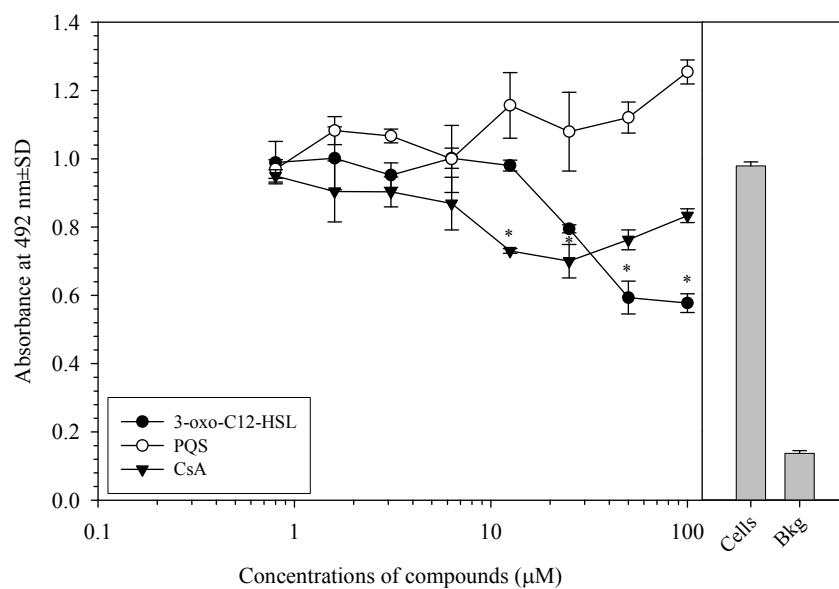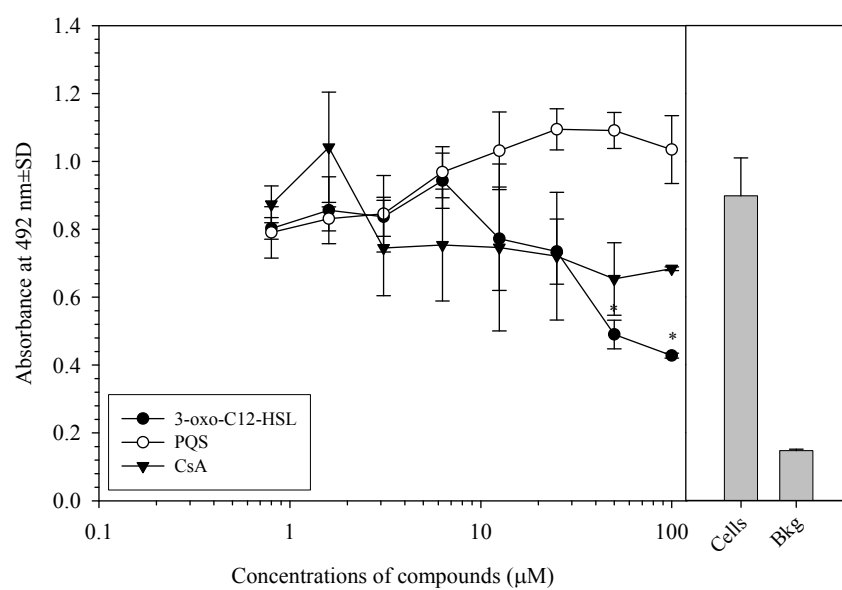
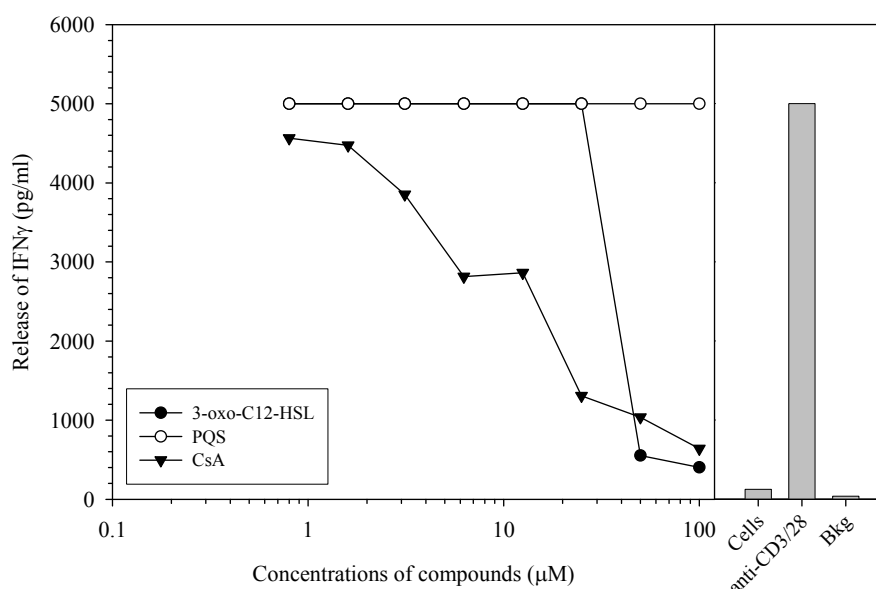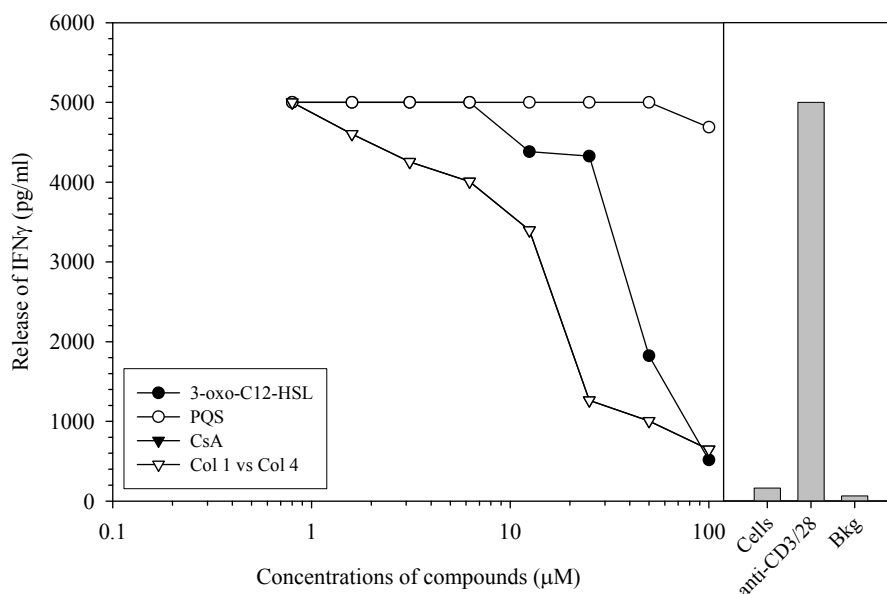**A.2.9. The effect of *P. aeruginosa* QSSMs and CsA on the release of IL-2 from human PBMCs.** ***P<0.001, **P<0.01, *P<0.05 compared to the anti-CD3 and anti-CD28 control. 'Cells' represents untreated cells and 'Bkg' (background) represents the measurements for CTCM.
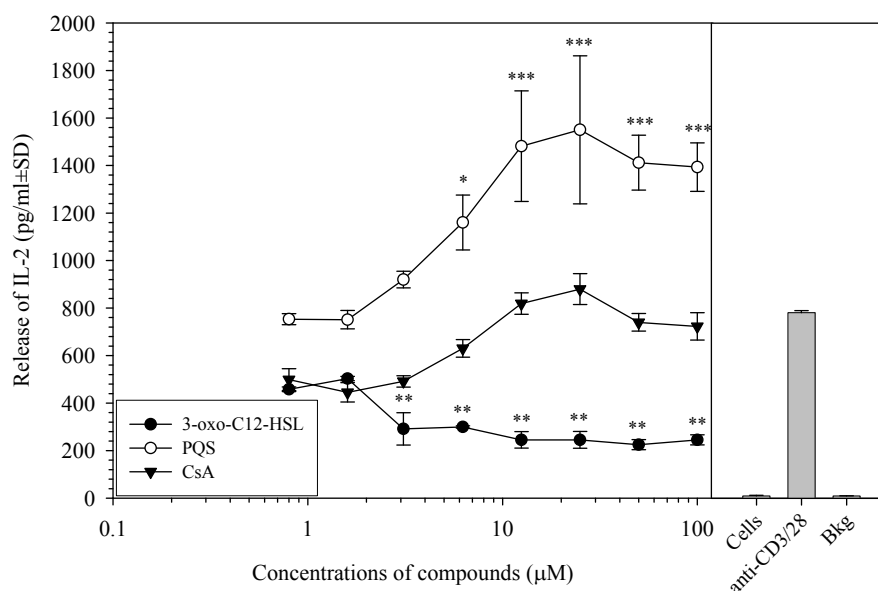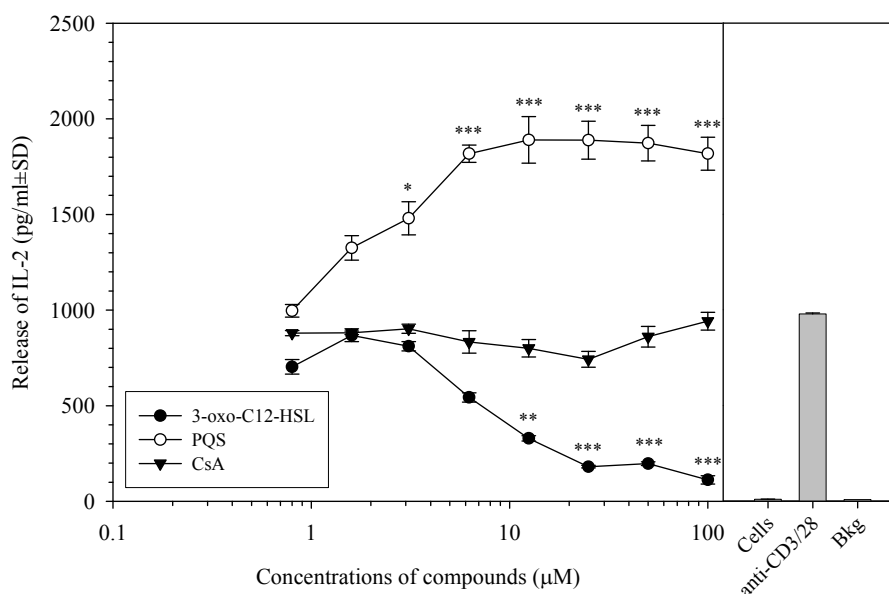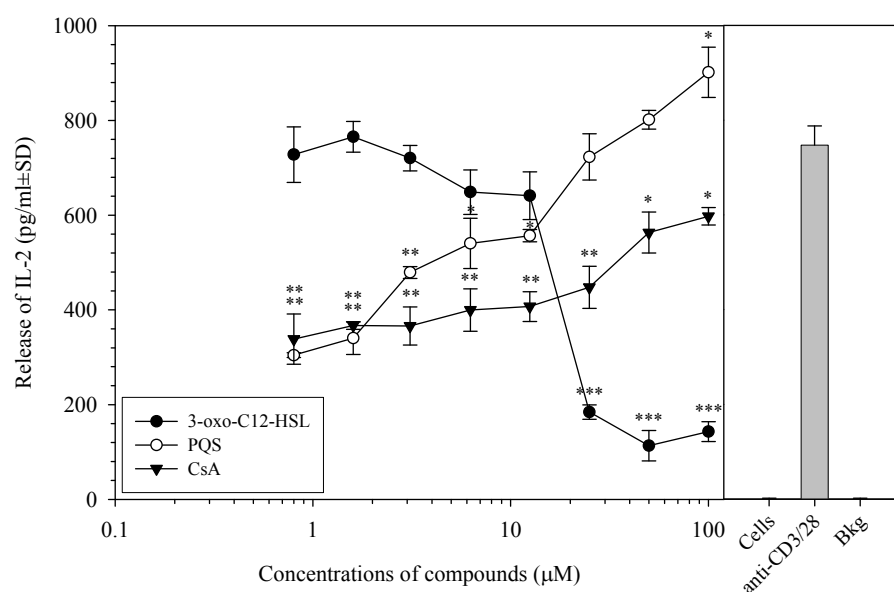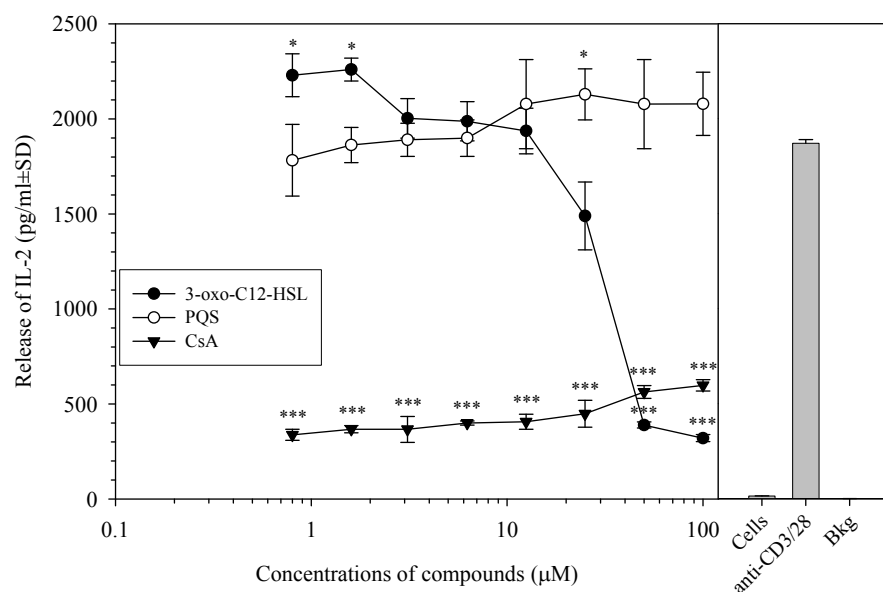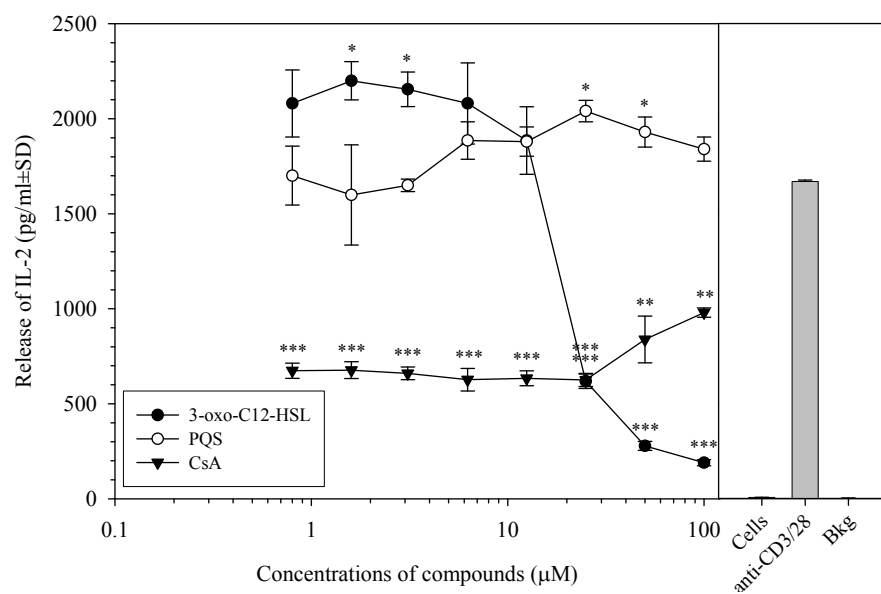
A.2.10. The effect of *P. aeruginosa* QSSMs and CsA on anti-CD3 and anti-CD28 driven pure T cell proliferation. ***P<0.001, **P<0.01, *P<0.05 compared with the anti-CD3 and anti-CD28 control. Cells represents untreated cells and Bkg (background) represents the measurements for CTCM.
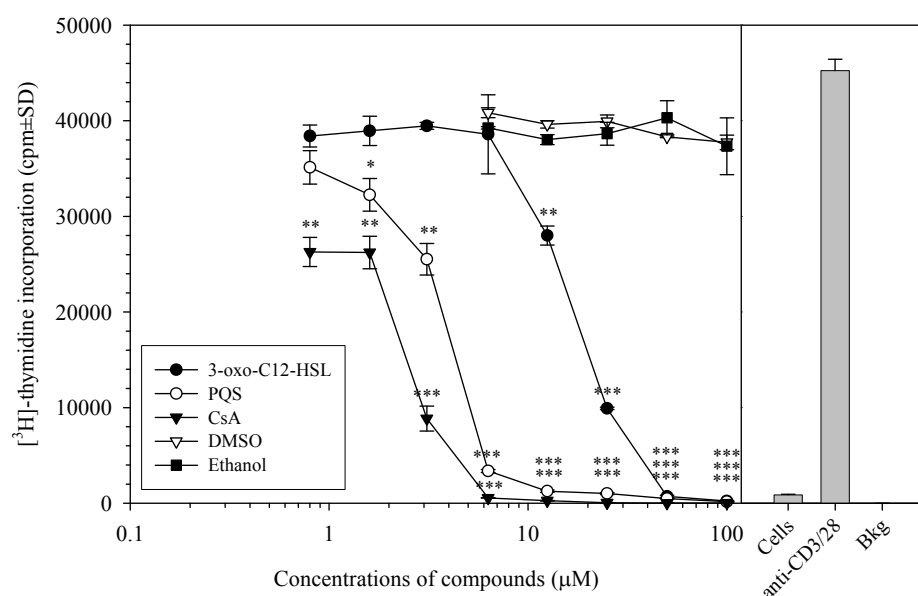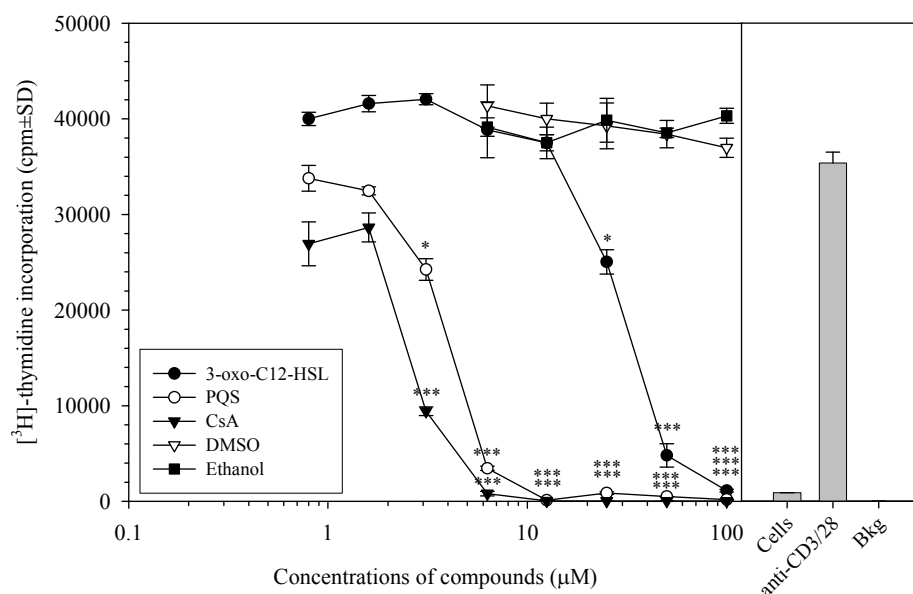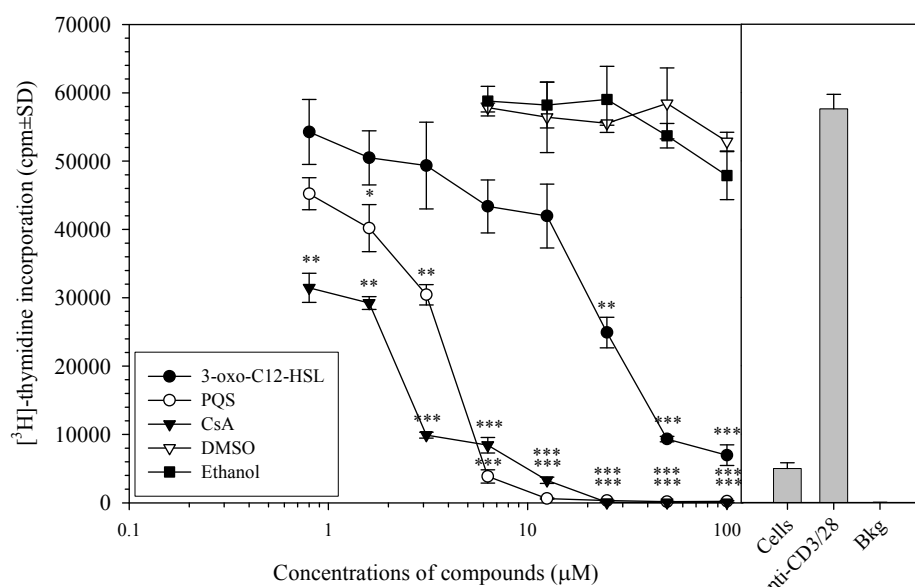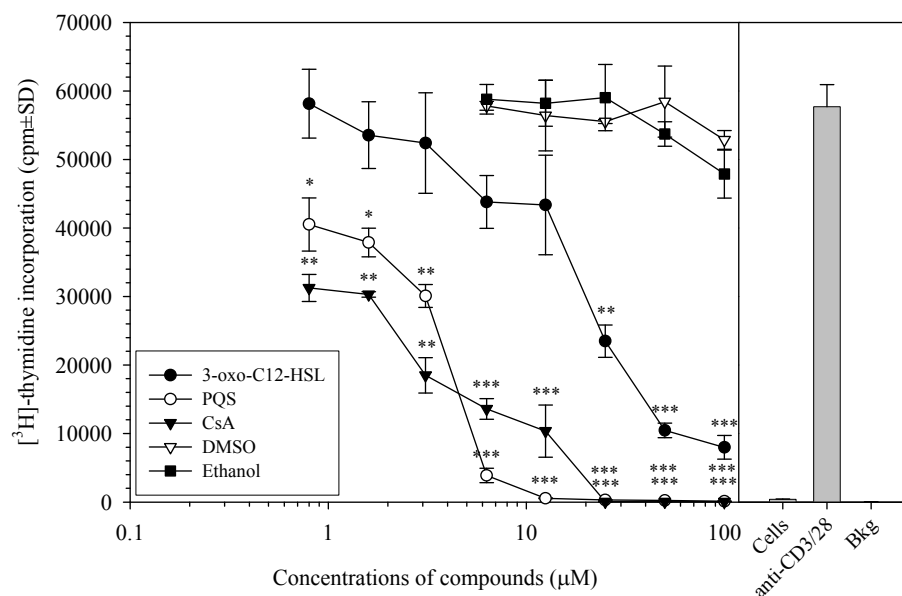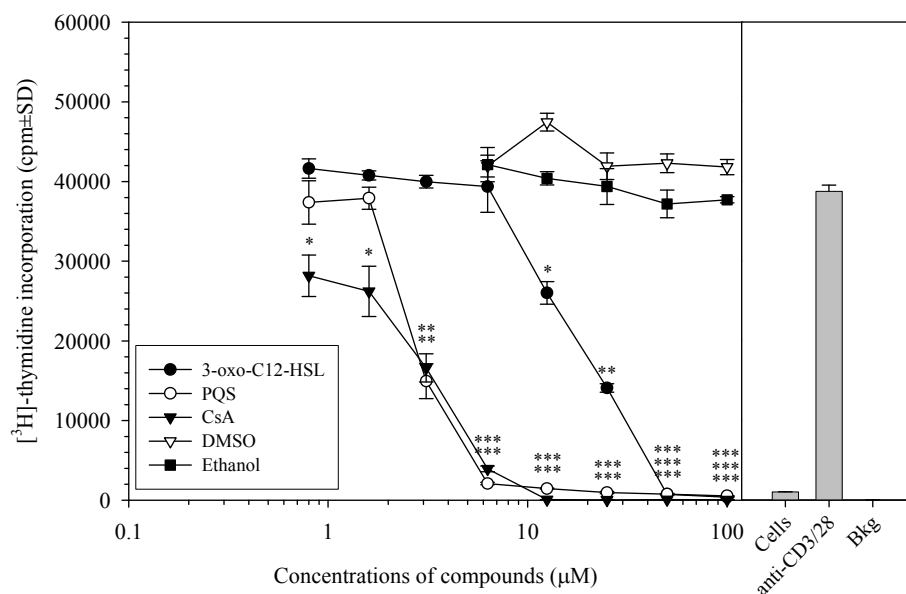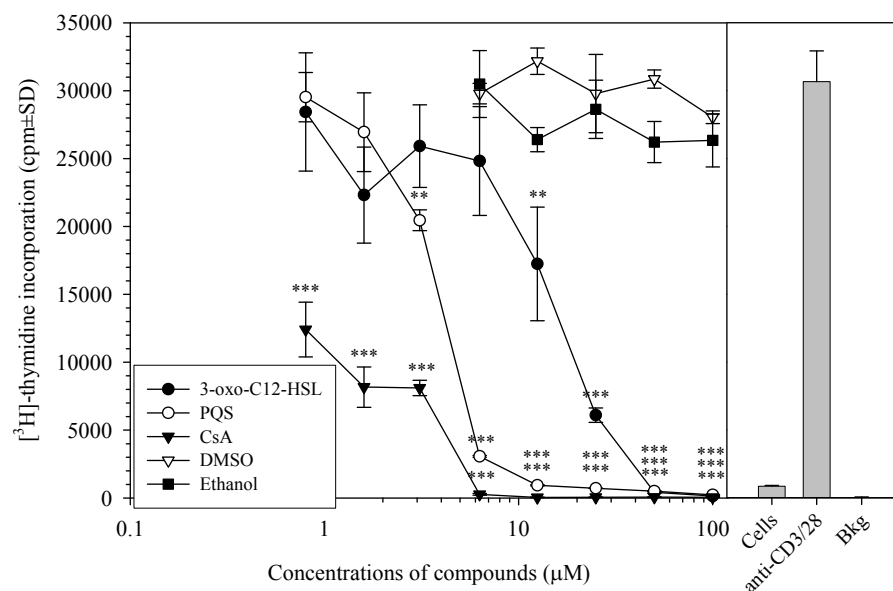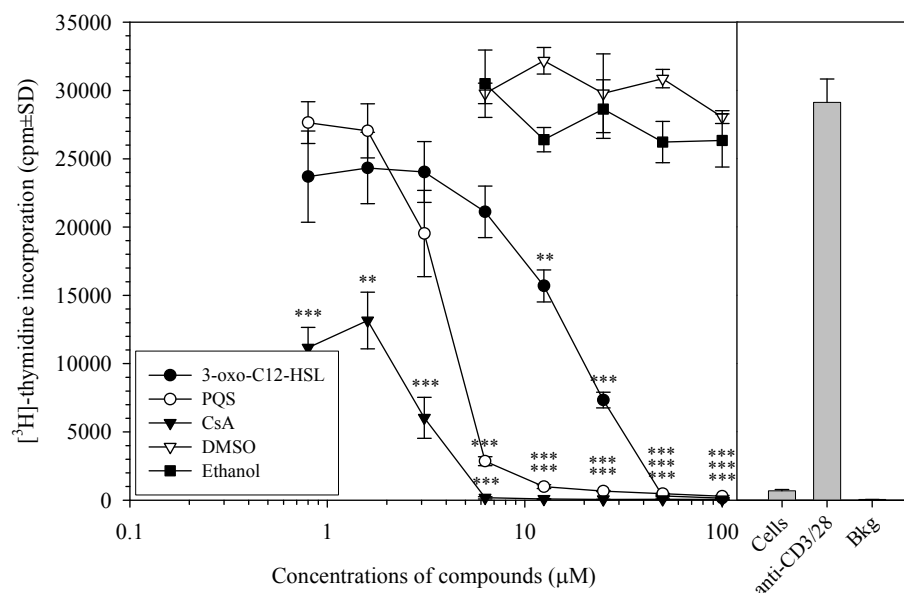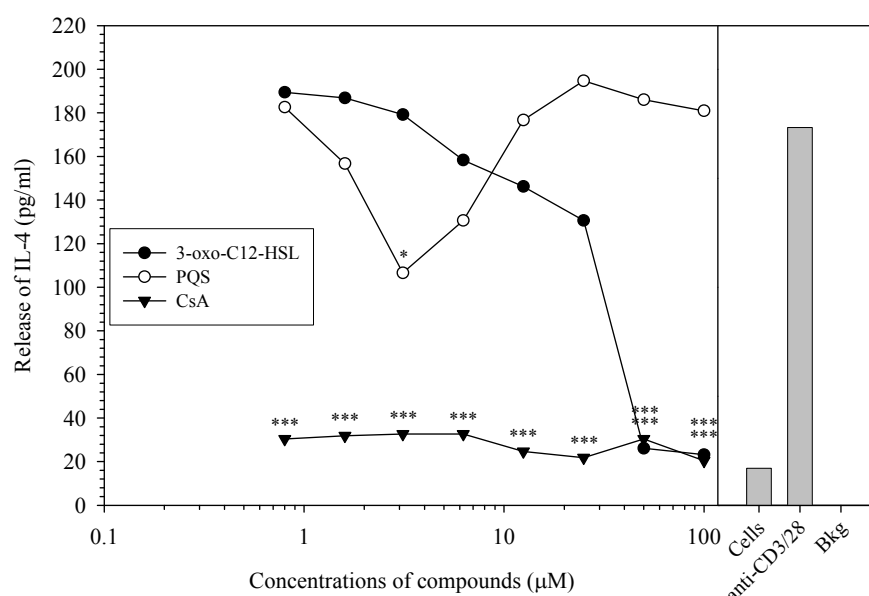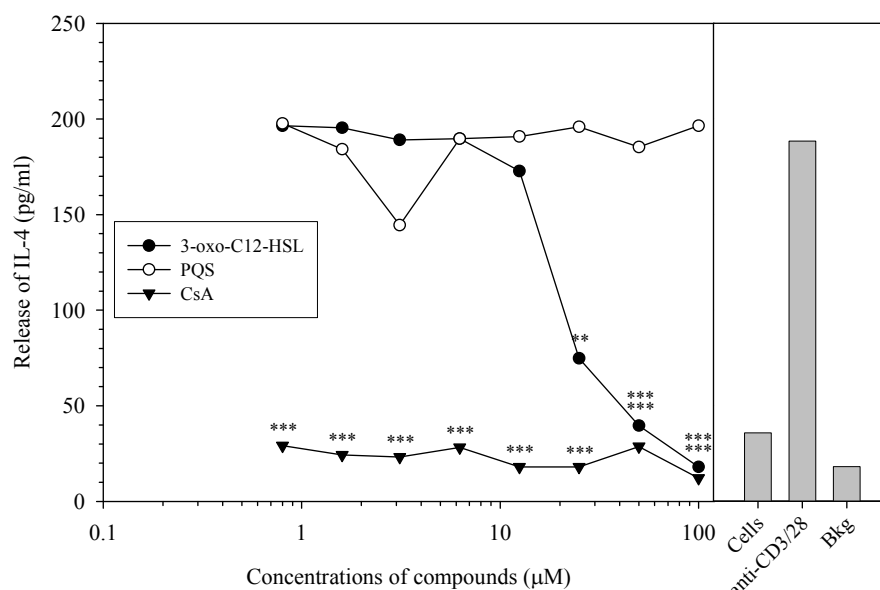
A.2.11. The effect of *P. aeruginosa* QSSMs and CsA on pure T cell viability.

*P<0.05 compared with the cell control (untreated cells). Bkg (background) represents measurements of CTCM.
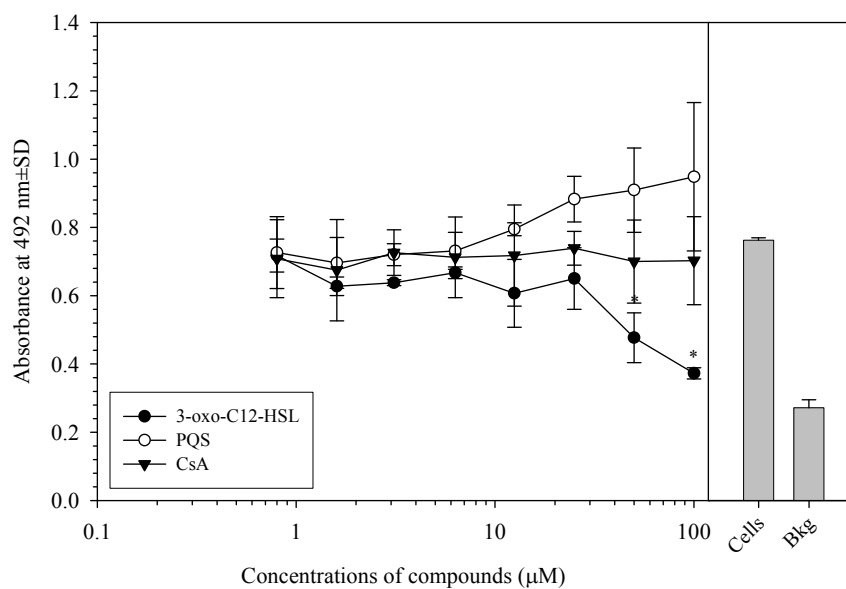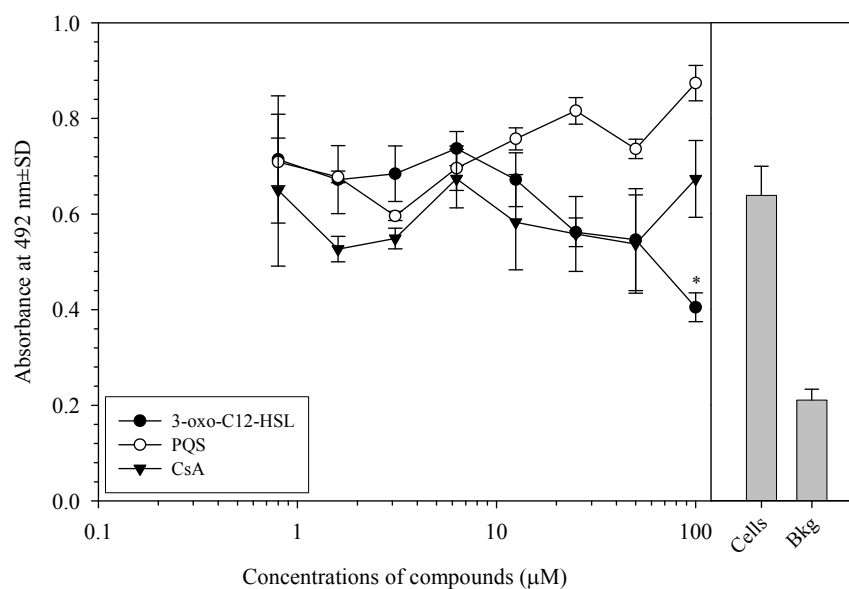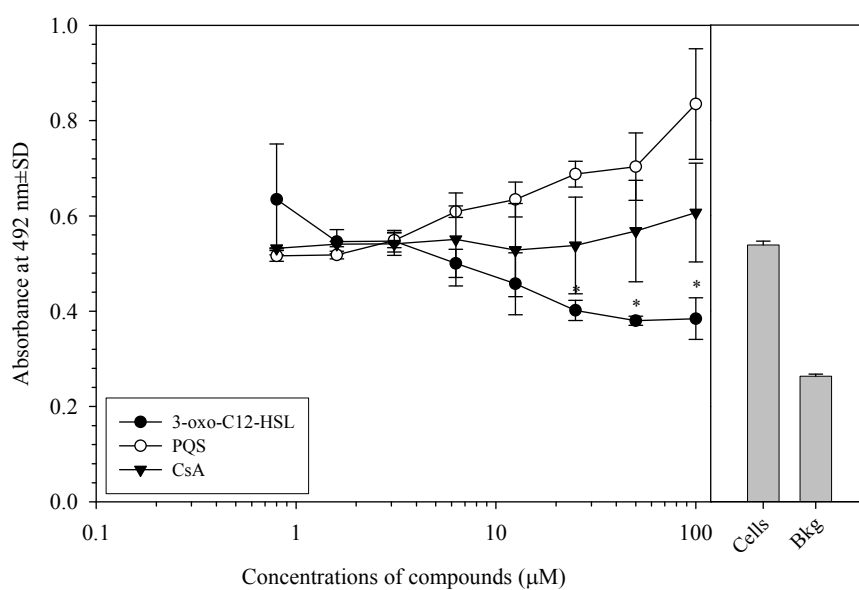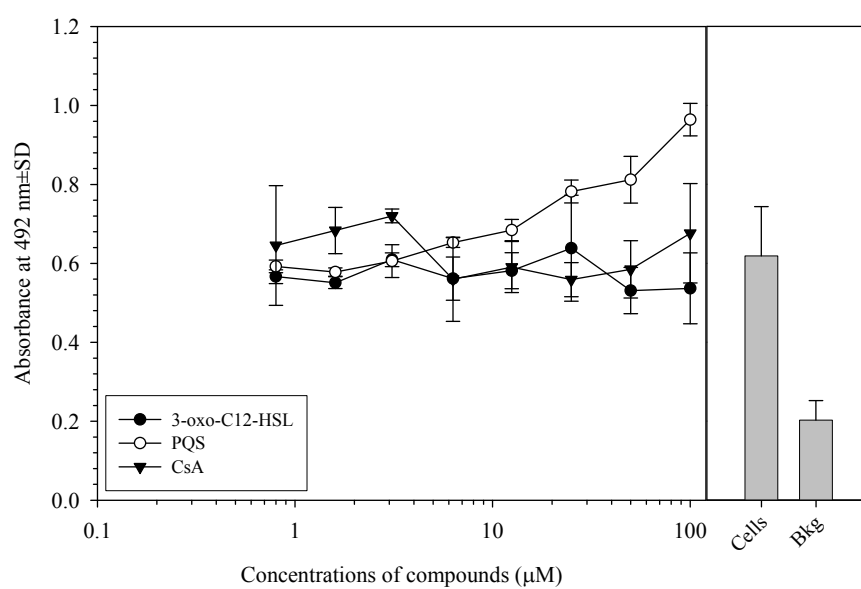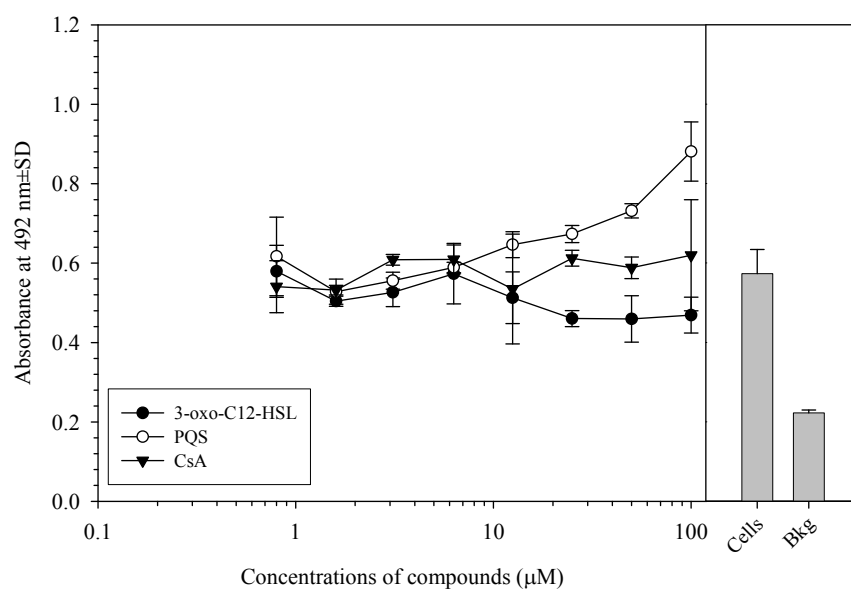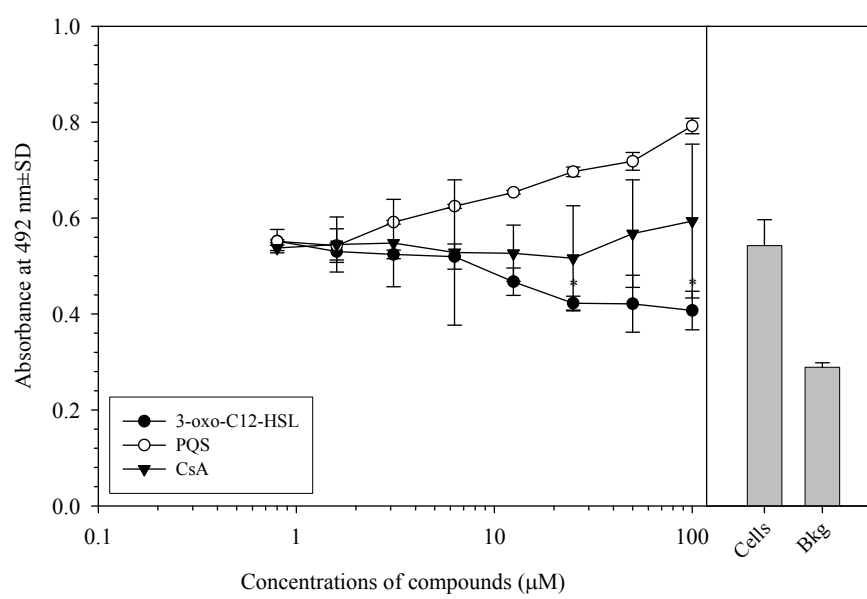
A.2.12. The effect of *P. aeruginosa* QSSMs and CsA on the release of IL-2 from pure T cells using the standard sandwich ELISA technique. ***P<0.001, **P<0.01, *P<0.05 compared with the anti-Cd3 and anti-CD28 control. Cell represents untreated cells and Bkg (background) is the measurement for CTCM.
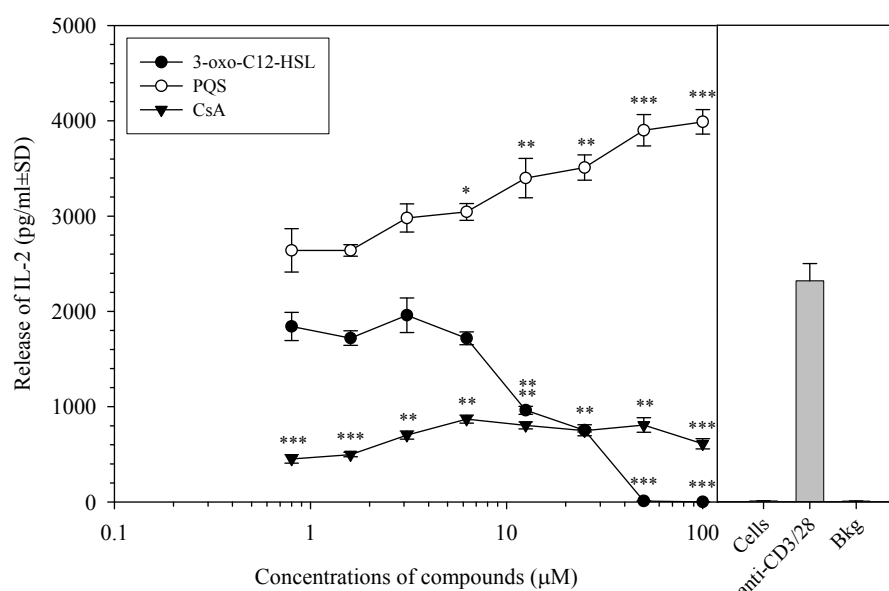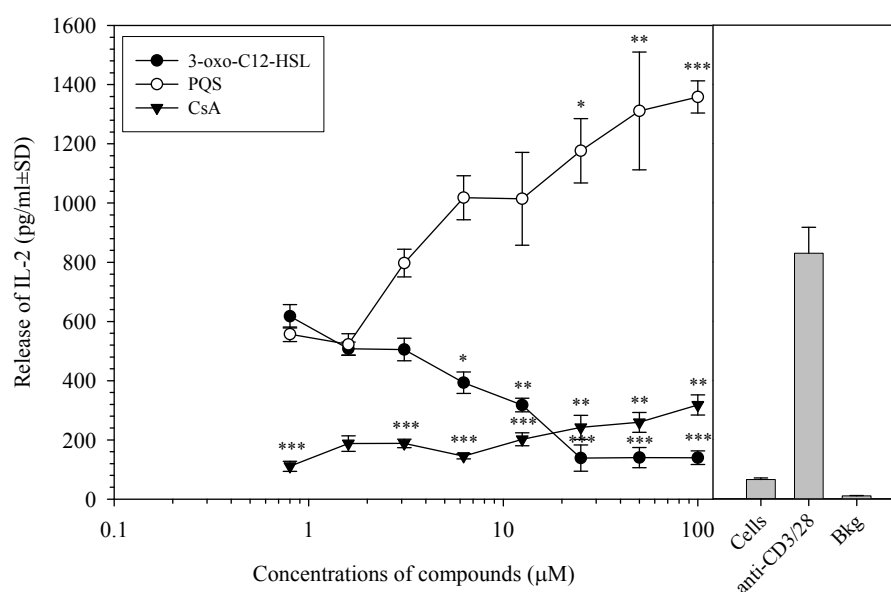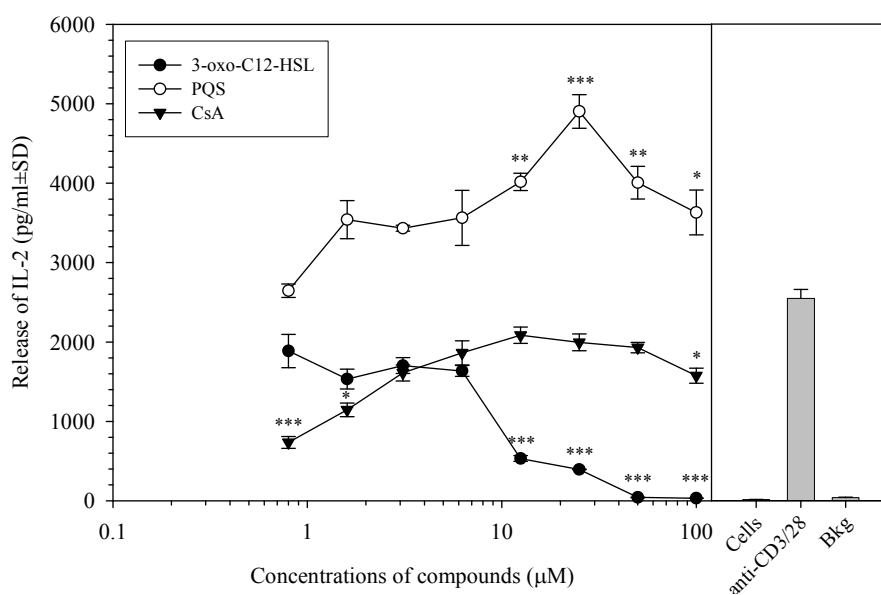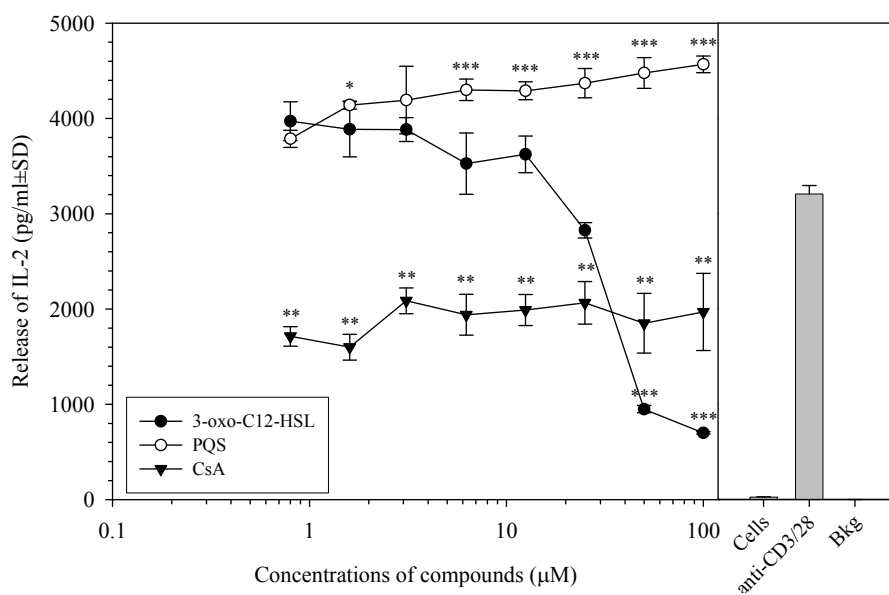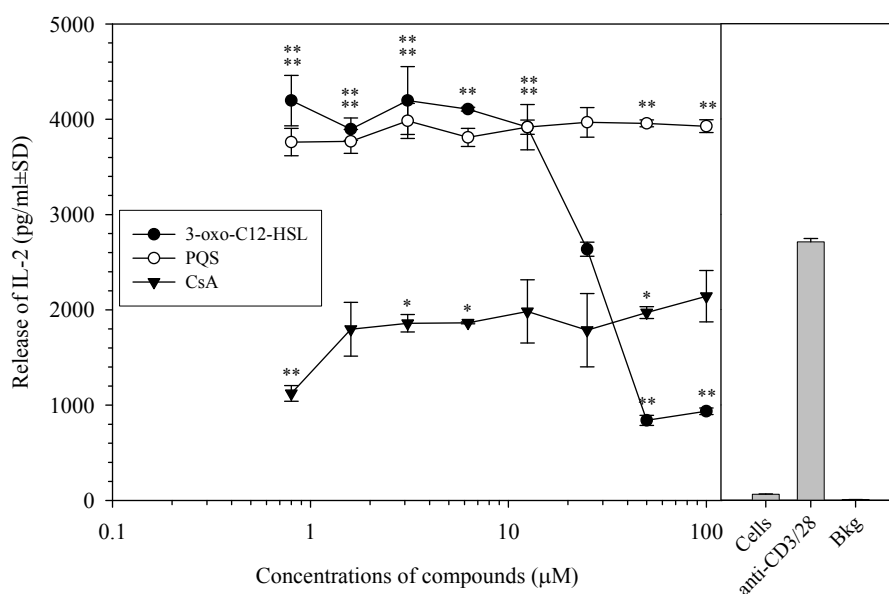
A.2.13. The effect of *P. aeruginosa* QSSMs and CsA on the release of IL-2 from pure T cells using cytometric bead analysis. ***P<0.001, **P<0.01, *P<0.05 compared with the anti-CD3 and anti-CD28 control. Cells represents untreated cells and Bkg (background) is the measurement of CTCM.
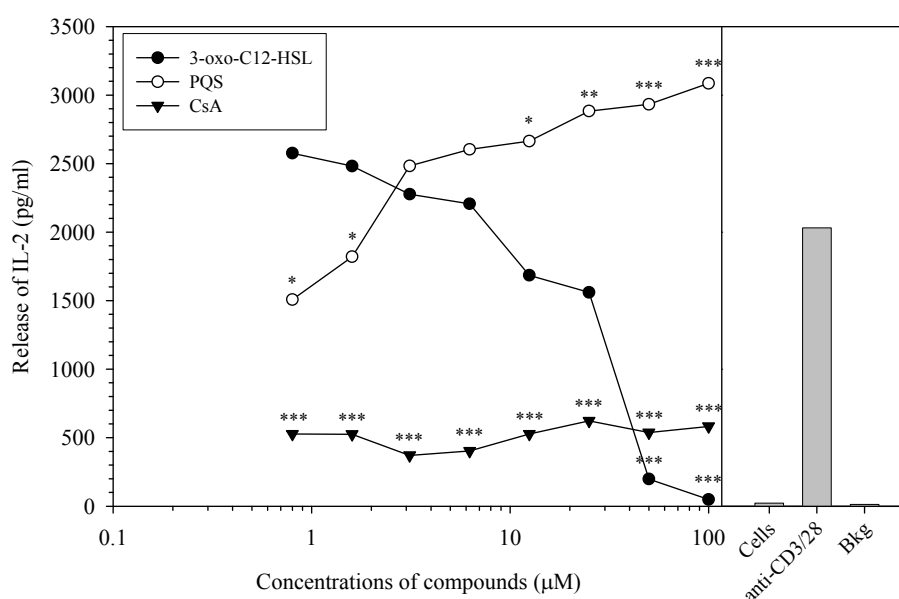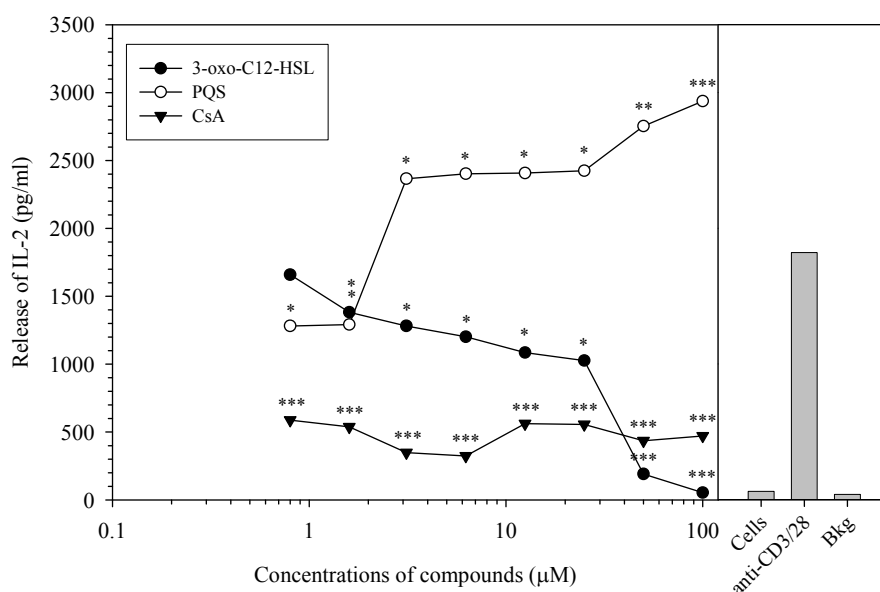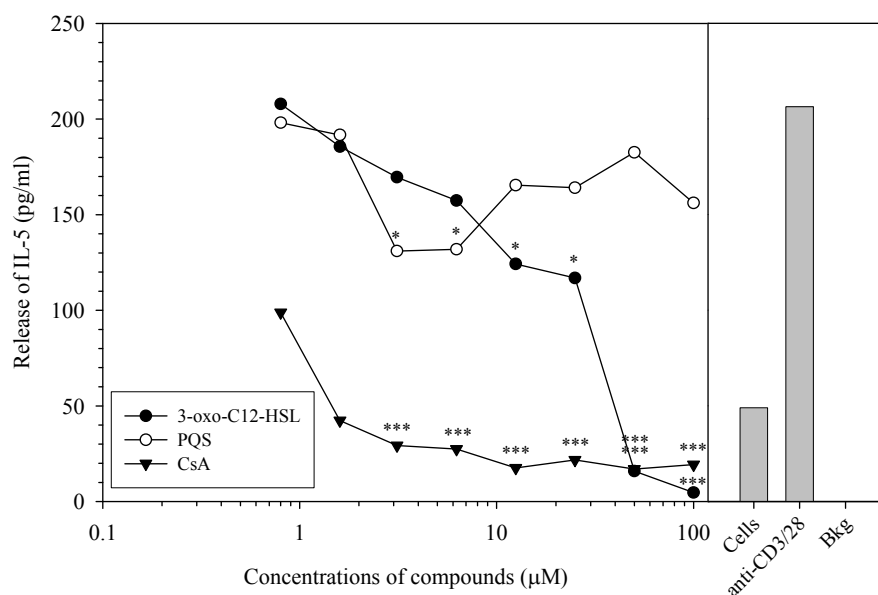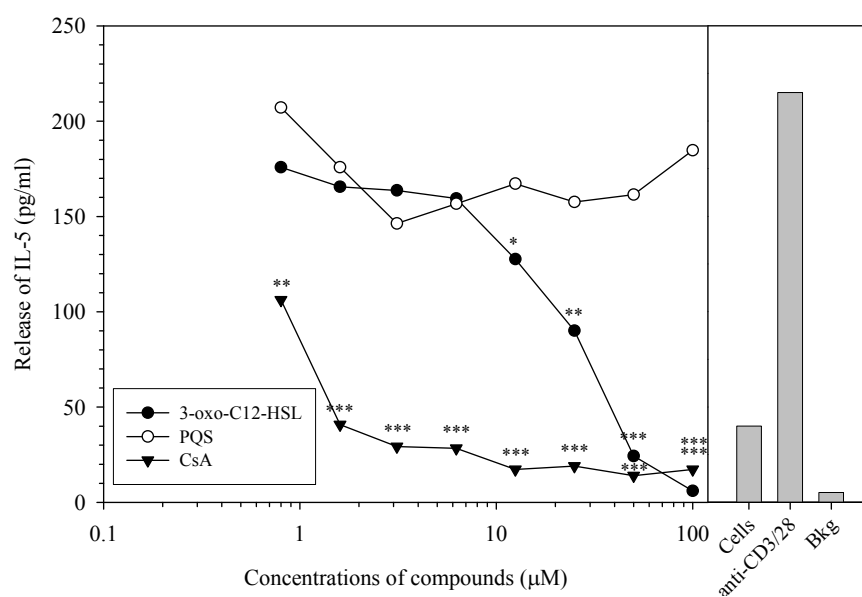
A.2.14. The effect of *P. aeruginosa* QSSMs and CsA on the release of IFNγ from pure T cells using cytometric bead analysis. Anti-CD3 and anti-CD28 control represents cells treated with only these two antibodies. Cells represents untreated cells and Bkg (background) is the measurement of CTCM.
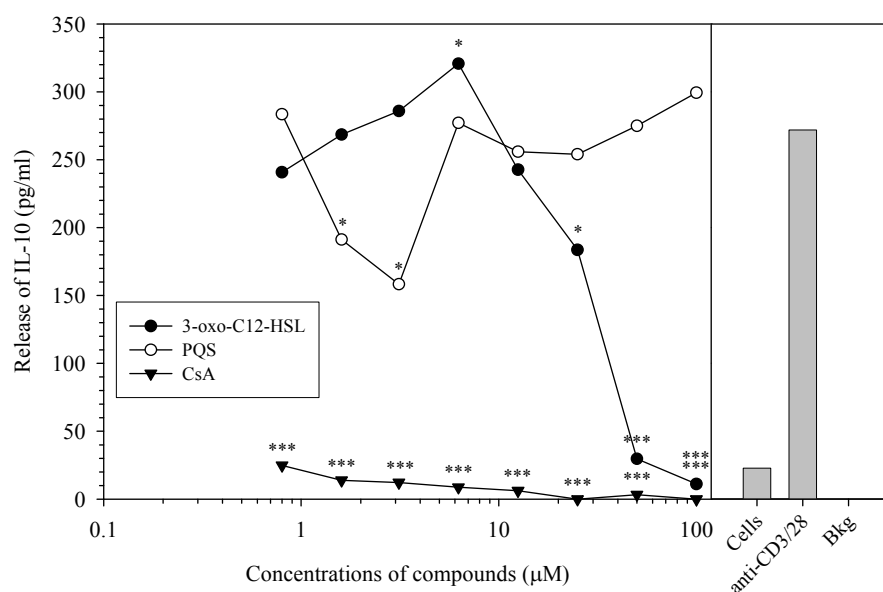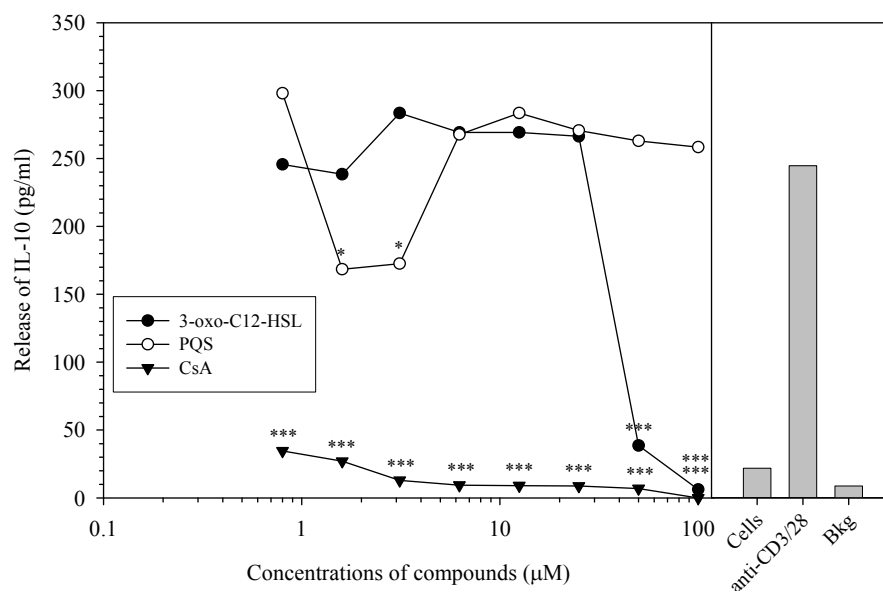
A.2.15. The effect of *P. aeruginosa* QSSMs and CsA on the release of IL-4 from pure T cells using cytometric bead analysis. ***P<0.001, **P<0.01, *P<0.05 compared with the anti-CD3 and anti-CD28 control. Cells represents untreated cells and Bkg (background) is the measurement of CTCM.

A.2.16. The effect of *P. aeruginosa* QSSMs and CsA on the release of IL-5 from pure T cells using cytometric bead analysis.   ***P<0.001, **P<0.01, *P<0.05 compared with the anti-CD3 and anti-CD28 control. Cells represents untreated cells and Bkg (background) is the measurement of CTCM.

A.2.17. The effect of *P. aeruginosa* QSSMs and CsA on the release of IL-10 from pure T cells using cytometric bead analysis. ***P<0.001, *P<0.05 compared with the anti-CD3 and anti-CD28 control. Cells represents untreated cells and Bkg (background) is the measurement of CTCM.

A.2.18. The effect of *P. aeruginosa* QSSMs and CsA on the release of TNFα from pure T cells using cytometric bead analysis. ***P<0.001, **P<0.01, *P<0.05 compared with the anti-CD3 and anti-CD28 control. Cells represents untreated cells and Bkg (background) is the measurement of CTCM.