Effects of cl-CD95L during *in vitro* *Toxoplasma gondii* infection

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Infection and Global Health Medicine

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Declaration

I confirm that the work in this dissertation is my own work and contain no material that has been submitted for any other degree at the University of Liverpool or other institution.

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AAM- Alternatively Activated Macrophages
AIDS- Acquired Immune Deficiency Syndrome
Arg-1- Arginase
BMDM- Bone Marrow-Derived Macrophages
CAM- Classically Activated Macrophages
CNS- Central Nervous System
gp-130- glycoprotein 130
IFN- γ - Interferon gamma
IL- Interleukin
iNOS- Induced Nitric Oxide Synthase
IRAK- Interluken-1 Receptor-Associated Kinase 1
IRF1- Interferon Regulatory Factor-1
JAK/STAT- Janus Kinase and Signal Transducer and Activator of Transcription
KO- Knockout
LDH- Lactate Dehydrogenase
MAPK- Mitogen-Activated Protein Kinase
MDSC- Myeloid-Derived Suppressor Cell
MMP- Matrix Metalloprotease
MyD88- myeloid differentiation primary response 88
NK- Natural Killer
NLRP-3- nucleotide-binding domain and leucine-rich repeat containing a pyrin domain 3
PAMPs- Pathogen Associated Molecular Patterns
PRR- Pattern Recognition Receptors
PVM- Parasitophorous Vacuole Membrane
SCID- Severe Combined Immune Deficiency
SLE- Systemic Lupus Erythematosus
SOCS- Suppressor of Cytokine Signalling
STAT- Signal Transducer and Activator of Transcription
S1P- Sphingosine-1-phosphate
T. gondii- *Toxoplasma gondii*
TH1- T Helper type 1
TH2- T Helper type 2
TLRs- Toll-like receptor
TNF- Tumour Necrosis Factor alpha

Abstract

Approximately 1-2 billion people are infected with *T. gondii* globally, making it one of the most successful protozoan parasites. With this in mind, we believe it is critical to better understand immunogenic ligands that may increase the pathogenicity of *T. gondii* during mammalian infections. Previous research on Systemic Lupus Erythematosus (SLE) demonstrated that cl-CD95L aggravates inflammation. Therefore, this study will delve into the role of cl-CD95L as an aggravating factor in the development of pathogenic *T. gondii* responses. To carry out this investigation VERO cells were used to deliver preliminary results on the impact cl-CD95L has on cell viability and cytotoxicity. Following on from this, bone-marrow derived macrophages (BMDM) were stimulated with cl-CD95L and infected with *T. gondii.* BMDM cultures were analysed to uncover through which pathways cl-CD95L may lead to an increase in parasitemia during infection. Finally, a Phospho-Kinase Array was utilized and determined that cl-CD95L amplifies protein expression in non-infected BMDM. Interestingly, the proteins expressed are similar to those of a myeloid-derived suppressor cell (MDSC). From the data collected we believe that cl-CD95L causes an MDSC like phenotype in previously un-polarized BMDM. The hallmark of MDSC activity is the increase in Arginase-1 expression which leads to the depletion of L-arginine, this in turn decreases the immune response against *T. gondii,* leading to an increase in parasite replication.

Chapter 1. Introduction

1.1. Toxoplasma gondii

Toxoplasma gondii is the causative agent of the disease toxoplasmosis, this obligate apicomplexan intracellular protozoan parasite is prevalent in most warm-blooded animals (Howe and Sibley, 1995). In humans most *T. gondii* infections are asymptomatic however as an opportunistic parasite it is capable of causing devastating effects frequently manifesting in the development of brain abscesses and encephalitis (Lehmann *et al.*, 2006) (J G Montoya and Liesenfeld, 2004). *T. gondii*, therefore, more commonly causes disease in congenitally infected infants and immune-compromised adults (Hill and Dubey, 2002).

There is a vast socio-economic impact of toxoplasmosis especially due to the cost of care for those with mental impairment and blindness (Roberts, Murrell and Marks, 1994). Routine testing of all pregnant women for *T. gondii* occurs in some European countries such as France while others still debate the cost-benefit of such mass screening (Yudin *et al.*, 2013).

1.2. Life history of *T. gondii*

T. gondii is tissue-cyst-forming and uses a prey-predator system in which it alternates between intermediate and definitive hosts for asexual and sexual reproduction. Not only can *T. gondii* be transmitted between intermediate and definitive hosts but also between intermediate hosts via carnivorism or even between definitive hosts (Robert-Gangneux and Dardé, 2012).

Approximately 30-40% of domestic cats (*Felis catus*) are infected with *T. gondii* (Webster, 2010). Felidae family members are the definitive hosts of *T. gondii* and sexual reproduction of the parasite occurs in the intestine of the cat (Dubey, Lindsay and Speer, 1998). Figure 1 illustrates the lifecycle of *T. gondii* through its definitive and intermediate hosts while also demonstrating different routes of infection.

Bradyzoites invade the epithelial cells of the small intestine where they initiate the formation of numerous asexual generations before the sexual cycle begins. Bradyzoites are a slow multiplying stage that undergo a self-limiting number of asexual multiplications within enterocytes, this is characterised by the development of merozoites (Dubey, 1998). Sexual development then follows with the formation of both male and female gametes, which fuse and fertilise each other (Ferguson, 2002). Oocysts are then formed after fertilisation, within enterocytes, these are subsequently liberated from the cell and excreted as the unsporulated form in cat faeces (Jones and Dubey, 2010). Following sporulation in the environment, sporozoites become infective. Upon the intake of sporulated oocysts by an intermediate host, the sporozoites transform into the invasive

tachyzoite stages (Dubey, Lindsay and Speer, 1998). The tachyzoites are crescent-shaped approximately 2-6µm in width and 4-8µm in length, with a pointed anterior (apical end) containing secretory organelles and a rounded posterior end (Halonen and Weiss, 2013). Locomotion of *T. gondii* occurs via gliding motility, which is instrumental in parasite migration and cellular invasion (Sibley, Hâkansson and Carruthers, 1998). Tachyzoites actively penetrate the host cells through the cell membrane or by phagocytosis. After entering the host cell, tachyzoites are surrounded by a parasitophorous vacuole (Mordue *et al.*, 1999). Tachyzoites asexually replicate rapidly by repeated endodyogeny in non-intestinal epithelial cells of the definitive host and any cell of the intermediate host (Dubey, Lindsay and Speer, 1998). The rates of both invasion and growth differ depending on the strain of *T. gondii* (Appleford and Smith, 1997). There are three distinct clonal genotypes of *T. gondii*, type I, II, and III (Howe and Sibley, 1995). Type I has been shown to be virulent in mice regardless of the host genotype and in humans as seen in acquired immune deficiency syndrome (AIDS) patients. Whereas, type II is non-virulent to mice while still seen as virulent in humans and commonly associated with congenital infections and in patients with AIDS (Howe *et al.*, 1997). Finally type III is non-virulent in mice but still virulent in other animals although it is less commonly seen in humans (Sibley *et al.*, 2009). After the repeated rounds of replication, the host cells are exited and the tachyzoites invade neighbouring cells. This host cellular destruction is responsible for the clinical manifestations of the parasite (Tenter, Heckeroth and Weiss, 2000).

Bradyzoites multiply slowly within a tissue cyst, these tissue cysts grow and remain intracellular as the bradyzoites replicate by endodyogeny (Robert-Gangneux and Dardé, 2012). They are predominantly located in the central nervous system (CNS), cardiac and skeletal muscles as well as the eye due to their high affinity for neural and muscular tissues (Tenter, Heckeroth and Weiss, 2000). These cysts can break down, in turn, releasing bradyzoites. Therefore, in an infected animals' lifetime, the tissue cyst burden constantly evolves and may persist for life in some intermediate hosts (Figure 1) (Schlüter *et al.*, 2014).

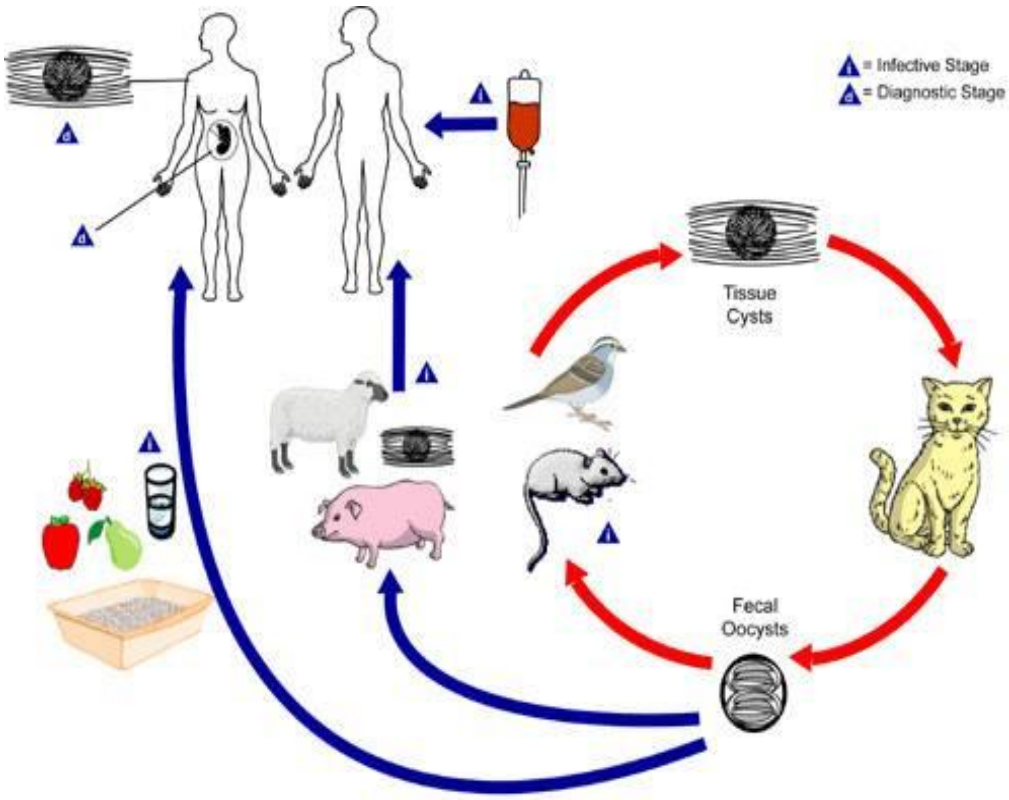

Figure 1. Lifecycle of *Toxoplasma gondii*. A diagram illustrating the lifecycle of *Toxoplasma gondii*, while also identifying both the infective and diagnostic stages (Adapted from the CDC, 2015).

The blue arrows show infection of humans by *T. gondii* where they act as an intermediate host. During pregnancy the parasite can be transmitted across the placenta, infecting the foetus. The red arrows show the infection and continued cycling of *T. gondii* through the definitive host, which is the cat (Adapted from the CDC, 2015).

1.3. Modes of Transmission

The life cycle of *T. gondii* may be able to continue indefinitely by the transmission of oocysts between definitive hosts but also by the transmission of tissue cysts between intermediate hosts (Tenter, Heckeroth and Weiss, 2000). *T. gondii* infections may be transferred between intermediate hosts including humans through the horizontal transmission of viable cysts in the food chain (Montoya and Liesenfeld, 2004). *T. gondii* tissue cysts have been found in a plethora of meat products from pigs, lambs and goats, and game (Cenci-Goga *et al.*, 2011). In Europe and also the USA the major source of *T. gondii* in humans is undercooked pork (Dubey, 1998).

Humans can also be infected by oocysts, which have been excreted from the cat through the faecal oral route. Oocysts can remain viable as they are resistant to external environmental conditions in soil and water (VanWormer *et al.*, 2013). The variations of seroprevalence of *T. gondii* have been found to correlate with hygiene and eating habits of a population supporting the oral route as a major source of infection (Cook *et al.*, 2000).

The vertical transmission of tachyzoites from mother to unborn foetus causes congenital toxoplasmosis. This occurs when the woman acquires a *T. gondii* infection for the first time during gestation. *T. gondii* will then enter the foetus by infection of the placenta (Remington, Thulliez and Montoya, 2004).

The frequency of *T. gondii* transmission increases with the gestational age at the point of infection. Consequences to the offspring are severe at the beginning of the pregnancy however the trans-placental passage of tachyzoites is rare (Robert-Gangneux and Dardé, 2012).

Congenital toxoplasmosis leads to approximately 4-10% of pregnancies resulting in stillbirths, abortion or neonatal deaths (Remington *et al.*, 2006). The incidence of congenital toxoplasmosis evaluated varies between different countries with the incidence in Brazil at 1 per 3000 live births and in France with 3.3 per 10,000 live births (Neto *et al.*, 2000)(Villena *et al.*, 2010).

1.4. Diagnosis, treatment and prevention

Toxoplasmosis has nonspecific clinical signs, which mimics a variety of other infectious diseases and therefore these alone cannot be relied upon for a definite diagnosis (Halonen and Weiss, 2013). Serologic examination can aid the diagnosis of a *T. gondii* infection. The most reliable serologic test is the Sabin-Feldman dye test which is used to detect antibodies to *T. gondii* (Sabin and Feldman, 1948). In the case of immunosuppressed or patients with AIDS, the diagnosis of toxoplasmosis is made by biopsy findings of *T. gondii* in host tissue, this is useful due to suppressed host antibody synthesis (Montoya, 2002).

Pyrimethamine is the most effective treatment for toxoplasmosis as it has been shown to kill the proliferating tachyzoites both *in vitro* and *in vivo*. A second drug such as sulfadiazine or trisulfapyrimidines should be used in combination with pyrimethamine for the most effective outcome in humans however they do not target tissue dwelling bradyzoites (Nath and Sinai, 2003). However, a review of 114 patients with Toxoplasma encephalitis found drug toxicity in 62% of the patients treated with pyrimethamine and sulfadiazine, 44% of which required a change in therapy due to severe side effects (Porter and Sande, 1992).

There is currently no human vaccine available to prevent infections in humans although there is a live vaccine that has been shown to reduce abortion in sheep (Rorman *et al.*, 2006). Toxovax is based on a live attenuated S48 strain, used in sheep across Europe and New Zealand, is unsuitable for human use due to the ability of the attenuated parasite to revert to a pathogenic strain(Kur, Holec-Gąsior and Hiszczyńska-Sawicka, 2009).

There is a plethora of public-health measures that can be implemented to reduce transmission and the disease burden of *T. gondii* in both humans and animals. The most important risk factor for human infection is known to be uncooked meat, therefore, all meat products should be cooked throughout to 56C for 10 minutes before human consumption. Raw meat should never be fed to cats due to the possible presence of tissue cysts while their litter trays should be cleaned daily and faecal matter disposed of correctly. While pregnant, cats and livestock should be avoided to limit transmission of *T. gondii* (Hill and Dubey, 2002). A European multicentre case-control study found 6-17% of primary *T. gondii* infections were due to soil contact. To reduce contact with soil that may contain cat faeces gloves should be used when gardening (Cook *et al.*, 2000). Oocysts are capable of remaining viable in water for extended periods of time while being resistant to freezing (Robert-Gangneux and Dardé, 2012). There have been several outbreaks of toxoplasmosis in humans linked to drinking contaminated water (Bowie *et al.*, 1997). It has been found that chemical and physical treatments applied during water treatment, including chlorination and ozone treatment, are not effective in killing oocysts in the water. However, It has been found UV treatment to be an effective method to inactivate oocysts in drinking water (Dumètre *et al.*, 2008).

1.5. Cell Biology

T. gondii mechanisms of target cell invasions are distinct from other intracellular microorganisms. Unlike most viruses and intracellular bacteria, *T. gondii* requires no host cell participation but actively penetrates the host cell (Carruthers, 2002). *T. gondii* is capable of invading a variety of host cells in what has been described as a three-step process involving the secretion of proteins from secretory organelles, the rhoptries, the micronemes and the dense granules at the apical tip of the parasite (Carruthers and Sibley, 1997).

Studies have shown in the absence of these organelles, the invasion is suppressed and as a result, a lower number of the intracellular parasites present (Huynh, Boulanger and Carruthers, 2014). The first phase is a gliding motility phase, the second is the formation of the moving junction and the third is the development of the parasitophorous vacuole (Dubremetz, 2007).

The gliding motility phase allows *T. gondii* to reach a host cell and make contact between the host cell membrane and its apical tip. Gliding motility is promoted by dynamic rearrangements of the cytoskeleton and actin-myosin interactions of the parasite (Carruthers and Boothroyd, 2007).

A moving junction is formed starting as initial close contact between the host cell membrane and the apical end of *T. gondii* that quickly turns into a ring encircling the parasite. Gliding backward allows entry of *T. gondii* into the host cell membrane, called the moving junction. For the establishment of the moving junction, an apical membrane antigen must be distributed across the surface of the invading parasite. *T. gondii* also requires the secretion of rhoptry neck

proteins, which are injected into the host cell to function as a receptor for apical membrane antigen on the parasite (Alexander *et al.*, 2005).

The moving junction relocates from the apical end to the posterior end of *T. gondii*, which leads to the internalization of the parasite into the parasitophorous vacuole. Dense granular proteins also contribute to the formation of the parasitophorous vacuole membrane (PVM) (Nichols, Chiappino and O'Connor, 1983).

During the invasion process, most host transmembrane proteins are stripped from the PVM. This process prevents fusion with lysosomes as it modifies the biochemical characteristics of the PVM. Thereby allowing the parasite to avoid destruction by phagolysosomes (Sinai, 2008). In intermediate hosts, tachyzoites divide by endodyogeny within the PVM, forming two daughter cells within each mother cell. After approximately 64 to 128 parasites have accumulated in the PVM they will exit the cell (Robert-Gangneux and Dardé, 2012). In the definitive host, once the female gamete is fertilised and the oocysts mature, they will be discharged into the intestinal lumen through the rupturing the epithelial cells (Jones and Dubey, 2010).

1.6. The Host immune response to *Toxoplasma gondii*

The first step in the host immune response is the detection of infectious non-self, which enables the host to mount a response against infection. Initiation of the innate immune response begins with the recognition of pathogen-associated molecular patterns (PAMPSs) by germline-encoded receptors, which are commonly expressed on immune cells called termed pattern recognition receptors (PRRs) (Mortaz *et al.*, 2017). There are several classes of PRRs including Toll-like receptors (TLRs), which are involved in the pathogenesis of diseases (Duffy and O'Reilly, 2016).

TLRs have been shown to have an important role in non-self-discrimination through sensing infection and initiating the innate and adaptive immune response (Yarovinsky, Hieny and Sher, 2008). TLRs are strongly associated with the pathogenesis of inflammatory and autoimmune diseases (Kawai and Akira, 2010). Adaptor proteins include myeloid differentiation primary response 88 (MyD88), which is essential for survival in parasite-infected animals whereas the loss of individual TRLs has a very little impact on survival (Scanga *et al.*, 2002).

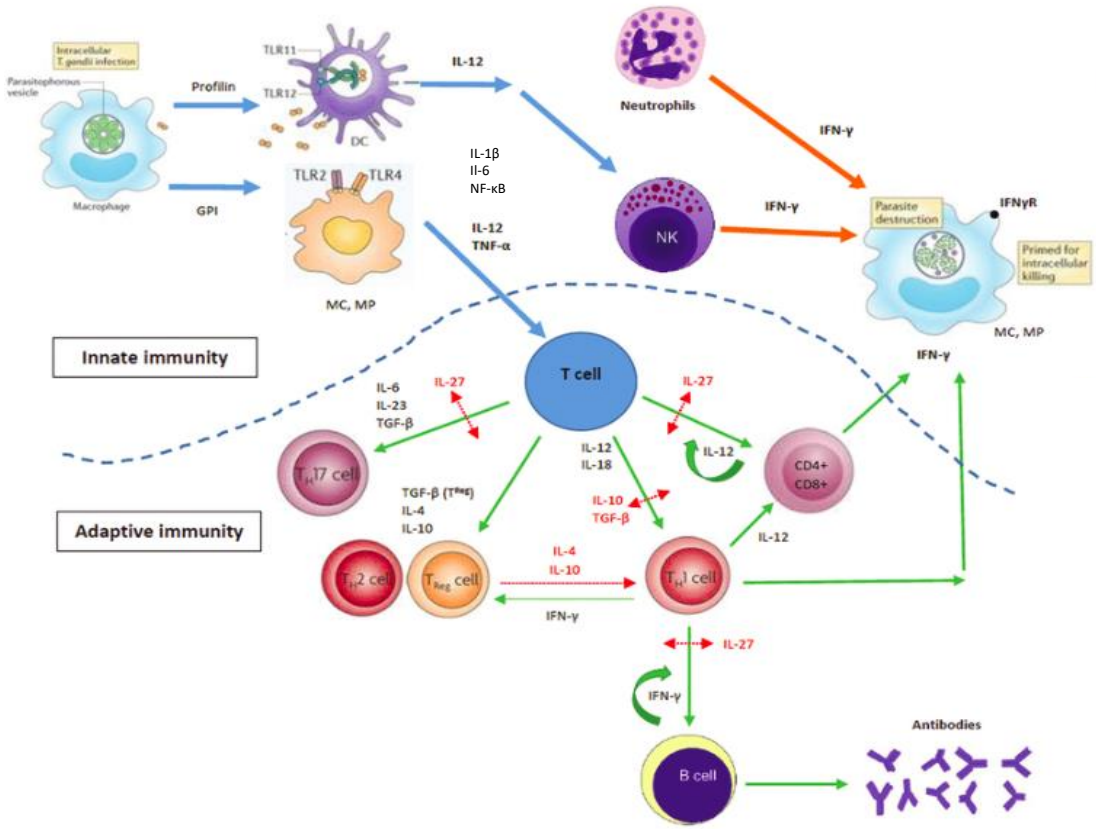

Figure 2. Summary of toxoplasmacidal pathways in humans (Adapted from Molan *et al.*, 2016)**.**

The classical stimulation pathway of TLRs activates nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPK) via the Interleukin-1 receptor-associated kinase 1 (IRAK) complex. The proinflammatory signalling pathway of TLRs activates the production of inflammatory cytokines and chemokines including IL-1β, IL-6 (Mortaz *et al.*, 2017). Figure 2 illustrates the pathways in which *T. gondii* activated the innate immune response and later the adaptive immune response through TLRs.

1.7. Innate immunity to *Toxoplasma gondii*

On initial infection, *T. gondii*, triggers the activation of macrophages and natural killer (NK) cells along with other haematopoietic and non-haematopoietic cells. Parasite proliferation will be limited by the host due to innate cytotoxic action (Filisetti and Candolfi, 2004).

To control the acute phase of *T. gondii* infection the early production of IL-12 by neutrophils, macrophages, and dendritic cells is crucial. A knockout (KO) of the *MyD88* gene has been shown to impair IL-12 production leading to increased susceptibility of mice to *T. gondii*. Due to uncontrolled parasite replication mice succumb to acute infection, dying within 13-16 days post infection in comparison to their control counterparts which survived >60 days post infection (Scanga *et al.*, 2002). IL-12 stimulates interferon-gamma (IFN- γ) secretion from NK cells and T-cells, which is crucial following infection by *T. gondii* (Khan, Matsuura and Kasper, 1994). Once IL-12 binds to its receptor this leads to the activation of the signal transducer and activator of transcription 4 (STAT4) signalling cascade, which is essential for IFN-γ production (Maier *et al.*, 2002). This has been confirmed in STAT4 KO mice, which are susceptible to acute infection with *T. gondii* due to the reduction of IFN- γ production (Cai *et al.*, 2000). The importance of IL-12 and its relationship with IFN- γ has also been shown by antibody neutralisation of IL-12, which results in 100% mortality of mice that were infected with *T. gondii*, and decreased production of IFN-γ was associated with this increased mortality (Gazzinelli *et al.*, 1994).

IFN- γ has a multitude of biological activities, which includes the activation of NK cells and macrophages (Filisetti and Candolfi, 2004). It has been suggested that high levels of IFN-γ are correlated with infection with virulent strains of *T. gondii* and increased apoptosis (Gazzinelli *et al.*, 1996). SCID mice possess an IFN-γ-dependent mechanism of resistance to intracellular *T. gondii*. This is dependent on the innate production of IFN-γ by NK cells, which is induced by IL-12 (Hunter, Chizzonite and Remington, 1995). Studies have shown IFN-γ KO mice have impaired IL-12 responses to *T. gondii,* in which the mice fail to control acute infection with mortality being associated with uncontrolled tachyzoites replication (Scharton-Kersten *et al.*, 1996).IFN-γ stimulation induces expression of p47 guanosine triphosphate family members, of which IGTP, LRG47, and IRG47 are essential for acute or chronic resistance to *T. gondii* respectively (Collazo *et al.*, 2001). Both IL-12 and IFN-γ direct Th1 differentiation during a primary immune response. T Helper type 1 (Th1) cells have also been shown to regulate their own response by producing IFN-γ in a positive feedback loop (Loddenkemper *et al.*, 2006).

Inducible nitric oxide synthase (iNOS) plays an important role in the primary pro-inflammatory response in macrophages upon pathogen infection (Wang *et al.*, 2009). There are two major pathways, which are known to influence iNOS gene expression within macrophages. These are the Janus kinase and STAT pathway, together known as the JAK/STAT pathway and the MAPK pathway. IFN-γ activates JAK/STAT pathway, which leads to the activation of the interferon regulatory factor-1 (IRF1) and the formation of phosphorylated STAT1 dimers that are key transcription factors in the regulation of iNOS transcription (Salim, Sershen and May, 2016). iNOS inhibition results in the suppression of the immune response against intracellular parasites (El Kasmi *et al.*, 2008).

Macrophages and mast cells produce tumour necrosis factor -α (TNF-α), which stimulates microbicidal activity against *T. gondii* in macrophages that have been primed by IFN-γ (Hunter, Chizzonite and Remington, 1995). TNF-α decreases intracellular levels of *T. gondii* by inducing lysosomal fusion of the parasitophorous vacuole (De Titto, Catterall and Remington, 1986). TNF-α boosts NO to act in a positive feedback loop, triggering iNOS-dependent *T. gondii* control (Masek and Hunter, 2013). After treating macrophages with anti-TNF- α antibodies and stimulating with IFN- γ, the production of reactive nitrogen intermediates are reduced along with toxoplasmcidial activity (Langermans *et al.*, 1992).

IL-6 is expressed by antigen presenting cells including dendritic cells and macrophages but it is also produced by a number of non- haematopoietic cells such as epithelial cells and keratinocytes. The production of IL-6 can be induced in response to external stimuli including IL-1 and TNF-α (Arango Duque and Descoteaux, 2014). IL-6 elicits a pro-inflammatory response when it binds to the heterodimeric receptor, glycoprotein 130 (gp130). During *T. gondii* infection IL-6 –mediated gp130 signalling blocks the generation of protective immunity (Silver *et al.*, 2011). Cytokines such as IL-6 have the capability to activate STAT3, which limits IL-12 production to prevent an infection-associated immune pathology, however, *T. gondii* uses the activation of STAT3 to evade the host immune response. Suppressor of cytokine signalling molecule 3 (SOCS3), which is a target of STAT3, has been shown to limit IL-6 signalling. SOCS3 is up-regulated during *T. gondii* infection, without the up-regulation of SOCS3, IL-6 signalling will not be suppressed which leads to the uncontrolled replication of *T. gondii* (Whitmarsh *et al.*, 2011). Dienz and colleagues have shown that IL-6 can modulate the Th1-Th2 balance towards T Helper type 2 (Th2) differentiation. IL-6 inhibits Th1 differentiation by directly up-regulating IL-1 production but also by inducing the expression of SOCS during the activation of $CD4^+$ T cells which interferes with IFN-γ signalling and production (Dienz and Rincon, 2009). IL-6 KO mice infected with *T. gondii* suffer from more severe tissue damage, increased incidence of Toxoplasmic encephalitis, and decreased IFN- γ levels (Suzuki *et al.*, 1997).

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IL-1β is a member of the interleukin-1 family of cytokines, which is mainly produced by monocytes and macrophages. The IL-1β cytokine family members are under transcriptional control, however, these cytokines differ from other cytokines in that they lack a leader sequence and they are expressed as biologically inactive preforms in the cytoplasm of cells (Latz, 2010). These cytokine preforms are the substrates of the cysteine protease caspase-1, which mediates the cleavage and release of the mature, biologically active cytokine forms. Caspase-1 itself is present as an inactive proform in the cytoplasm and it is activated by proteolytic self-processing (Thornberry *et al.*, 1992). Several multimolecular proteins complexes, referred to as inflammasomes, have been identified as caspase-1 activators, which are required of IL-1β cytokine activation (Latz, 2010).

IL-1β is a highly inflammatory cytokine and is also involved in a plethora of cellular activities, including cell proliferation, differentiation, and apoptosis (Netea *et al.*, 2015). IL-1β enhances T cell activation and antigen recognition and has been reported to induce the development of Th17 cells (van de Veerdonk and Netea, 2013). It has been shown during *in vitro Toxoplasma* infection of macrophages activates the nucleotide-binding domain and leucine-rich repeat containing a pyrin domain-3 (NLRP-3) inflammasome, resulting in the rapid production and cleavage of IL-1β (Gorfu *et al.*, 2014). *T. gondii* dense granule proteins GRA18, GRA27, and GRA28 are essential for the activation of NLRP1 inflammasome. The activation of the NLRP1 inflammasome in rats causes pyroptosis, preventing parasite replication within the host cell (Cirelli, 2016).

1.8. Classically and alternatively activated macrophages

Macrophages play a pivotal role in homeostasis and defence and are a widely distributed immune cell (Martinez *et al.*, 2008). Macrophages react to a wide variety of external stimuli and are able to produce a multitude of effector molecules for intercellular communication, microbial defence, and modulation of inflammatory reactions. They induce, suppress, or modulate both innate and adaptive immune responses (Rath *et al.*, 2014). M1/M2 describes the two major and opposing activities of macrophages. M1 cause tissue damage while actively inhibiting cell proliferation. On the other hand, M2 cause tissue repair and actively promotes cell proliferation. The names M1 and M2 were chosen because M1 and M2 macrophages promote Th1 and Th2 responses, respectively (Mills, 2012).

Macrophage metabolism of L-Arginine is an important parameter to identify classically and alternatively activated macrophages (Bronte and Zanovello, 2005). L-Arginine is an essential amino acid, which is used for the biosynthesis of proteins, creatine, and agmatine. It is metabolised by the enzymes iNOS and arginase 1 (Arg-1), which produces urea and L-ornithine, and nitric oxide and L-citrulline(Bronte and Zanovello, 2005). NOS and Arg-1 are competitively regulated by both Th1 and Th2 cytokines but also by complex intracellular biochemical pathways (Noël *et al.*, 2004).

The cytokine IFN-γ drives the classical pathway of macrophage activation and is produced by Th1 cells. IFN-γ increases NO release through NOS2 activity and inhibits IL-4- or IL-10-driven Arg-1 activity (Martinez and Gordon, 2014).

Cytokines produced by Th2 cells including IL-4, IL-10, and IL-13, regulate the alternative activation of macrophages. Each of these cytokines induces Arg-1 synthesis and inhibits NOS2 activity (Menzies *et al.*, 2011).

Murine infections with *Leishmania major* have demonstrated that in macrophages, a Th1 response induces NO synthesis and parasite killing, whereas Th2 cytokines inhibits NO synthesis and enables parasite growth. Supporting the idea that host arginase activity can promote parasite spread, macrophages were infected with *L. major* and treated with Th2 cytokines, which are all inducers of arginase. This lead to a proportional increase in the number of intracellular parasites (Modolell *et al.*, 2009).

T gondii induces NO production by macrophages, which is essential for parasite regulation. *T.gondii* infected Arg-1 KO mice had an improved ability to combat infection. However, *T. gondii* supresses NO production by utilising host arg-1 (El Kasmi *et al.*, 2008).

1.9. Adaptive immune responses

During the chronic stage of *T. gondii* infections, parasite latency is maintained by the adaptive T cell response. Both $CD4^+$ and $CD8^+$ T lymphocytes are involved in the host resistance against *T. gondii* infections (Filisetti and Candolfi, 2004). Athymic nude mice lack T cells and are therefore extremely susceptible to both virulent and avirulent strains of *T. gondii* (Langermans *et al.*, 1992).

Major histocompatibility complex (MHC) molecules are cell surface proteins where their function is to present antigenic peptides to T cells (Janeway *et al.*, 2001). *Toxoplasma* also interferes with the IFN-γ-induced MHC class I and MHC class II expression on the cell surface of bone marrow-derived murine macrophages (Luder *et al.*, 1998). Importantly, the inhibition of MCH class II expression by antigen-presenting cells influences $CD4^+$ T cell activation and proliferation (Munoz, Liesenfeld and Heimesaat, 2011).

$CD4^+$ T helper cells are crucial regulators of the adaptive immune response and inflammatory diseases. Antigen-specific $CD4^+$ T cells are activated by antigen-presenting cells and then differentiate into specialised effector cells that secrete cytokines (Dong, 2006)(Gazzinelli *et al.*, 1992). $CD4^+$ T lymphocytes can be categorised into the sub-populations, Th1, Treg, Th17, and Th2. The differentiation of naïve CD^+ T cells into Th1 cells is promoted by IL-12 (Salim, Sershen and May, 2016). Th1 cells produce IFN-γ and are key in cell-mediated immunity due to their ability to activate phagocytes and the production of opsonizing and complement-fixing antibodies (Santarlasci *et al.*, 2013). *T. gondii* infections cause a strong Th1 immune response with high levels of IFN-γ and TNF-

α, which activate macrophage functions. This is important for the control of tachyzoite replication during acute and chronic phases of infection (Denkers and Gazzinelli, 1998). In mice, chronically infected with an avirulent stain of *T. gondii*, treatment with anti-IFN-γ antibodies caused reactivation of infection leading to death of the all animals. Similar results were also seen in mice treated with anti-$CD4^+$ showing that both $CD4^+$ and IFN-γ prevent reactivation of latent infections (Gazzinelli *et al.*, 1992).

Regulatory T cells (Treg) have been shown to play an important role in establishing and maintaining immunological tolerance. However, they also have a negative impact on suppressing antitumor responses (Halim *et al.*, 2017). Human Tregs have been characterised by their expression CD25, IL-2 receptor and the transcription factor FoxP3 (Ziegler, 2007). Studies have shown that Treg cells mediate the T-cell suppression observed during acute *T. gondii* infection through an IL-2-dependent mechanism (Tenorio *et al.*, 2011). Following a *T. gondii* infection, the highly Th1 cell-polarized immune response was associated with a collapse in the number of Treg cells via multiple pathways, including the blockade of Treg cell induction and disruption of endogenous Treg cell homeostasis. In particular, the shutdown of IL-2 production contributed to Treg cell incapacity to suppress effector responses and eventually led to immunopathogenesis (Oldenhove *et al.*, 2009).

Th17 cells were identified as a new subpopulation of T helper cells that secrete IL-17 (Yao *et al.*, 1995). Th17 cells are inhibited by the cytokines produced by Th1 and Th2 cells, IFN-γ and IL-4 (Tesmer *et al.*, 2008). Th17 cells produce cytokines and transcription factors that can have both beneficial and pathogenic effects (Bailey *et al.*, 2014). Th17 cells and IL-17 expression has been associated with many inflammatory diseases in humans, such as systemic lupus erythematosus (Tabarkiewicz *et al.*, 2015). Following a *T. gondii* infection an increase in IL-17 production has been found to contribute to fatal *T. gondii* associated inflammation (Guiton *et al.*, 2010). However, studies have also shown in IL-17 KO mice, their susceptibility to *T. gondii* infection increases and display a high parasite burden and a low neutrophil influx, resulting in increased mortality from toxoplasma infection (Kelly *et al.*, 2005).

1.10. CD95 and its ligand CD95L

CD95 is a member of the tumour necrosis factor (TNF) receptor superfamily, which is expressed in a wide range of tissues (Dunay and Sibley, 2010). CD95 has an essential role in the elimination of cells that have been infected and ensuring immune tolerance to self-antigens (Tauzin *et al.*, 2011). However, the expression of its ligand (CD95L), which is a type II transmembrane protein of the TNF family, is limited to certain cell types including macrophages and T-cells (Li-Weber and Krammer, 2002). When membrane-bound CD95L binds to CD95, the intracellular region of CD95 orchestrates the formation of a death-inducing signalling complex. This is through the recruitment of the adaptor molecule, Fas-associated protein with death domain (FADD), which in turn induces caspase-8 aggregation and subsequent apoptosis (Khadra *et al.*, 2011). The expression of CD95L is tightly controlled, being expressed primarily at the surface of activated NK cells, macrophages, and T cells. However, CD95L can also be found at the surface of macrophages, epithelial cells or dendritic cells under inflammatory conditions (Penna *et al.*, 2012). Failure of CD95/CD95L signalling leads to an increased risk of cancer and autoimmunity (Tauzin *et al.*, 2011). The ligation of CD95 also plays critical roles during infection due to the activation of an apoptotic cascade. Parasite-induced apoptosis decreased significantly in CD95L KO mice. *T. gondii* causes cell resistance to a plethora of apoptotic inducers, in particular the parasite significantly reduces CD95-mediated apoptosis. It has been found that *T. gondii* blocks CD95-triggered apoptosis by caspase 8 inactivation however, the exact molecular mechanism through which *T. gondii* does so awaits clarification

(Vutova *et al.*, 2007). During infection with *T. gondii*, studies demonstrated that when using RH strain there were higher levels of apoptosis of the spleen in comparison to avirulent strains however; the proportion of infected cells did not rise above 2%. This suggests that the parasite is inducing apoptosis rather than an immunopathological basis causing programmed cell death (Gavrilescu and Denkers, 2001).

The extracellular domain of CD95L comprises a juxta-membrane stalk region, which is cleavable by metalloprotease, this cleavage releases CD95L into the bloodstream. cleaved-CD95L (cl-CD95L) promotes the formation of a motility-inducing signalling complex, which stimulates non-apoptotic signalling pathways and increases the levels of intracellular calcium content, leading to cellular migration (Poissonnier *et al.*, 2016).

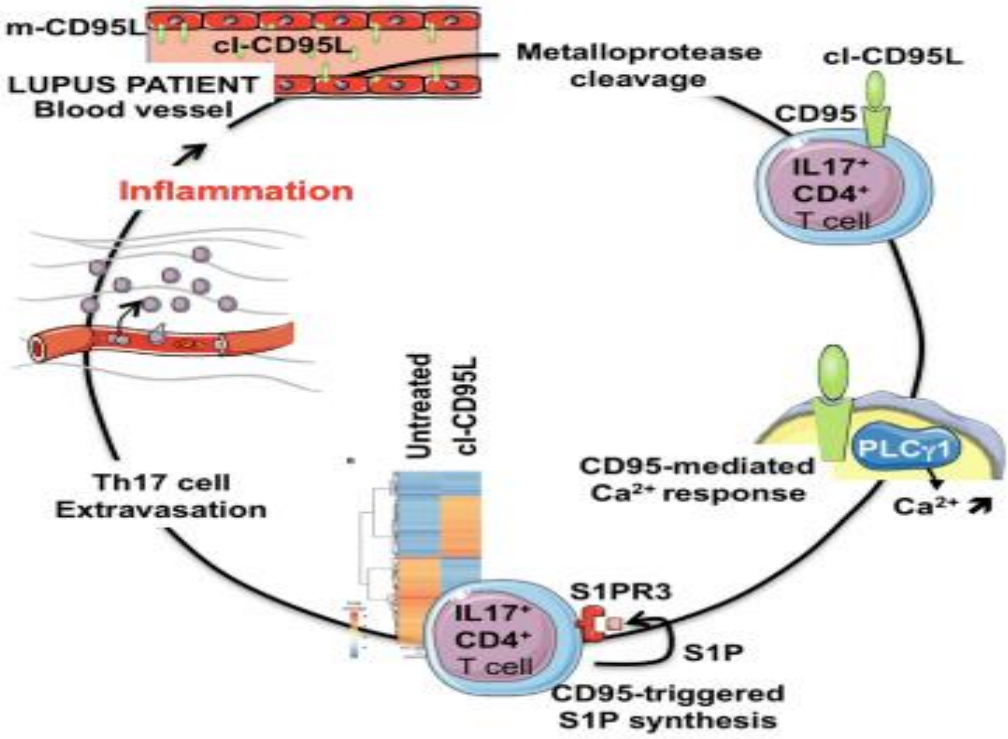

Figure 3. Summary of the pathogenic functions of cl-CD95L. Diagram illustrating the tight positive-feedback amplification loop for the pathogenic functions of cl-CD95L (Poissonnier *et al.*, 2016)**.**

Figure 3 illustrates the inflammation amplification loop caused by the cleavage of CD95L and the mechanisms that allow it to be an aggravating factor in Lupus patients. Mechanistically, as cl-CD95L binds to CD95, PLCγ1 is triggered and there is a rise in $Ca2^{+}$ signals promoting the endothelial transmigration of Th17 cells at the expense of the T regulatory cells (Poissonnier *et al.*, 2016). Furthermore, Th17 cells are found to up-regulate the expression of PSGL-1 adhesion molecule when exposed to cl-CD95L. PSGL-1 is known to promote lymphocyte and endothelial tethering and also can provoke secretion of effector cytokines in differentiated T cells (Figure 3) (Baaten *et al.*, 2013).

cl-CD95L has been found to aggravate the inflammatory process in systemic lupus erythematosus (SLE) patients, a chronic autoimmune disorder, whose pathogenesis can affect almost all organs and tissues. This happens by the recruitment of Th17 cells into organs that are inflamed. The abnormal migration of Th17 cells to inflamed organs promotes SLE pathogenesis and tissue destruction through immune complex deposition (Poissonnier *et al.*, 2016). Abnormal or elevated levels of cl-CD95L could be an aggravating factor in the development of pathogenic *T. gondii* specific T-cell responses where Th17 cells are involved.

1.11. Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) are a unique component of the immune system regulating immune responses in both healthy individuals and as part of various diseases (Gabrilovich and Nagaraj, 2009). MDSCs are rare in a healthy individuals however, may occur naturally in cases of sepsis or trauma to suppress the T-cell response (Tamadaho, Hoerauf and Layland, 2017). MDSCs have also been found to accumulate in the setting of many tumours and are associated with poor clinical outcomes and prognosis (Lechner, Liebertz and Epstein, 2010).

MDSCs are a heterogeneous population of myeloid origin (Gabrilovich and Nagaraj, 2009). MDSCs are composed of myeloid cells, immature myeloid cells, immature granulocytes, dendritic cells and monocytes-macrophages (Greten, Manns and Korangy, 2011). They may be further subdivided into two major types, firstly $CD11b^{+}Gr^{-}1^{low}$ monocytic MDSC and $CD11b^{+}Gr^{-}1^{high}$ granulocytic MDSC (Youn and Gabrilovich, 2010).

Several different factors have been found which can influence the expansion and activation of MDSCs including IL-6 vascular endothelial growth factor (VEGF-CSF), granulocyte/macrophage CSF (GM-CSF) and prostaglandins (Talmadge and Gabrilovich, 2013). These trigger the STAT3 pathway, STAT3 signalling has been implicated in the stimulation of myeloid cell differentiation into MDSCs. STAT3 interacts with CCAAT-enhancer-binding protein β (C/EBPβ). C/EBPβ has a key role in myeloid development, as C/EBPβ-deficient bone marrow cells lose the ability to differentiate into functional MDSCs (Thornberry *et al.*, 1992)(Zhang *et al.*,

2010). The activation of immature myeloid cells during pathological conditions leads to the expression of their immune suppressive factors to be upregulated (Barnie *et al.*, 2017). The combined effect of inducible iNOS and arginase 1 mediated local arginine deletion leads to the expansion of MDSCs (Lechner, Liebertz and Epstein, 2010). MDSCs are best known for their capacity to inhibit T-cell proliferation. The metabolism of L-arginase has been linked to the suppressive activity of MDSC as well as mature macrophages (Dilek *et al.*, 2012). MDSC will actively migrate to the site of a tumour where they will upregulate arginase 1 and iNOS expression while ROS production is downregulated. The levels of both NO and arginase 1 which are produced by a tumour associated MDSCs are higher than those found in the peripheral lymphoid organs of the same animal (Gabrilovich and Nagaraj, 2009) NO produced by MDSCs suppress T-cell function by the inhibition of JAK3 and STAT5 functions in T cells, the inhibition of the expression of MHC class II and inducing apoptosis of T-cells (Nagaraj and Gabrilovich, 2012). In an acute infection iNOS KO mice, which are unable to produce NO, control parasite growth at the site of inoculation. However, iNOS KO mice eventually succumb to chronic *T. gondii* infections showing that NO production is essential for long-term control of the parasite (Scharton-Kersten *et al.*, 1997). In MDSCs the increased activity of arginase 1 leads to the enhanced catabolism of L-arginine. This causes a depletion of L-arginine in the microenvironment, which inhibits T-cell proliferation (Geiger *et al.*, 2016).

1.12. Research Rationale

To date there has been no previous research conducted on the impact of cl-CD95L during a parasitic infection. However, other studies have demonstrated that cl-CD95L leads to non-apoptotic signalling pathways which have been shown to be an aggravating factor in autoimmune inflammation, mediated by Th17 responses. Therefore, the objective of this study is to investigate if cl-CD95L creates a favourable host niche for *T. gondii* and the consequences of this for the subsequent immune response. This understanding of the immune environment created during infection could facilitate future success in host-directed therapeutics in the treatment of *T. gondii*.

1.13. Aims

The aims of this project are therefore to:

1. Investigate cl-CD95L in relation to altered *Toxoplasma gondii* burden.
2. To investigate if cl-CD95L alters cellular responses after *Toxoplasma gondii* interaction.
3. To investigate if cl-CD95L causes an MDSC-like phenotype in BMDM.

Chapter 2. Materials and Methods

2.1. Vero cell cultures

Vero cells are a commonly used cell line which were originally isolated from kidney epithelial cells extracted from an African green monkey (Rhim and Schell, 1967). VERO cells were cultured in DMEM supplemented with 5% foetal bovine serum (FBS, F4135 Sigma Aldrich), 1% penicillin-streptomycin (10,000 U penicillin & 100 mg/ml streptomycin (P4333 Sigma Aldrich) at 5% CO_2 at 37°C. VERO cells were cultured in 25cm^2 flasks until 80% confluent. To do this, growth media was removed to allow cells to be washed with 5 mLs D-PBS. After which, 1 ml of 0.25% trypsin EDTA (25200-072, gibco life technologies) was added to the flask. The flask was then incubated at 37oC for 5 minutes. 10 ml of DMEM was added to inactivate the effects of trypsin. The cell suspension was transferred into a 15 ml tube and centrifuged at 2,000rpm for 10 mins. The supernatant was removed, and the pellet was resuspended in 10 ml of DMEM. The number of VERO cells were counted with haemocytometer using the trypan blue exclusion. 5 mLs of DMEM was added to new 25 cm^2 flask and seeded with $4x10^5$ VERO cells per 25 cm^2 flask.

2.2. Parasite cultures

The RH strain of *Toxoplasma gondii* was used throughout the work. Parasites were propagated as tachyzoites by serial passage in VERO cells cultured in DMEM supplemented with 5% FCS, 1% penicillin-streptomycin at 5% CO_2 at 37°C.

Parasites were harvested and then purified for use in experiments or re-inoculated into VERO cells every 7 days. For *T. gondii* purification host cells were scraped from flasks with rubber disposable cell scraper. The cells were passed, 3 times, through a 20mL syringe attached to a sterile blunt-ended needle. Cells were centrifuged for 10 minutes at 2000rpm at room temperature. PD-10 Columns were prepared by removing the bottom plug and draining the storage buffer from the column. The column was washed with 5mL falcon tube. Parasites were resuspended in 5mL 1X D-PBS. 5mL of parasite solution was added to the column, then washes through with another 5mL PBS. The number of parasites was counted in 10 µL of filtered parasite solution. Finally, the parasites were centrifuged for 10 minutes at 2,000rpm, supernatant removed and resuspended in DMEM at $1x10^5$/well.

2.3. Percentage monolayer destruction and free parasite counts

VERO cells were seeded into 24 well plates (Corning 3524) at $2.5x10^4$/well and incubated for 24 hours. Thereafter cells were stimulated with cl-CD95L at 200, 100 and 50ng/mL. 24 hours later cells were infected with *T. gondii* at $1x10^5$/well. After 72 hours post infection the percentage of the VERO monolayer that has been destroyed was estimated using an Olympus BH2 microscope at X10 magnification. An aliquot of supernatant was used to determine the extracellular parasite load.

2.4. Bone marrow-derived macrophages (BMDM)

Bone marrow-derived macrophages were obtained from healthy wild-type C57BI/6 mice aged between 8-16 weeks. Animals were culled by standard schedule 1 procedures of exposure to rising concentrations of CO^2. Immediately afterwards hind femurs were removed and placed into sterile PBS for transport to the laboratory. Excess fat, muscle, and skin were removed. Thereafter bone marrow was flushed from the femur by repeated injecting, using a 2mL syringe and a 25-gauge needle, of the femur with complete media. Cells were passed through a 70µm cell strainer and centrifuged for 10 minutes at 1,500rpm. Cells were re-suspended, and a cell count performed using Trypan Blue (Trypan Blue Solution, 0.4%, ThermoFisher Cat No. 15250061). Bone marrow cells were seeded at $2x10^6$/mL in a 6mL petri dish with the addition of 20ng/mL of M-CSF

(Recombinant Murine M-CSF, PeproTech Cat No. 315-02). Every two days change media/M-CSF was replenished until cells were harvested on day 7.

To harvest BMDM, media was first removed, and cells washed with PBS. Using PBS and a cell scraper, all cells were gently removed from the petri dish. Cells were collected by centrifuging at 1,200rpm for 5 minutes. Cells were resuspended and counted using Trypan Blue exclusion, thereafter BMDM were seeded in 96well flat-bottomed plates (CELLSTAR® Tissue Culture Plates, Greiner Bio-One, Cat No. 655180) at $2x10^4$/well.

2.5. BMDM stimulation and infection

After BMDM were seeded, the cells were stimulated with cl-CD95L at 200, 100 and 50ng/mL. 24 hours later cells were infected with *T. gondii* at $1x10^5$/well. After 48 hours post infection the supernatant was taken off and the cell lysis buffer (Triton X-100, VVR International Ltd, Product no. 28817 at 0.1% in PBS) was added to the BMDM then both were frozen at -80^oC. An aliquot of supernatant was used to determine the extracellular parasite load.

2.5.1 Pre-exposure of BMDM

Before stimulation with cl-CD95L and infection with *T. gondii* BMDM were exposed to different inhibitors.

To inhibit IL-6 production, anti-mouse IL-6 (Bio Cell Cat No. BE0046) was used at 120 ng/mL. Anti IL-6 was added during stimulation with cl-CD95L and the again 24hrs later during *T. gondii* infection. For the inhibition of Arginase, N^{ω}-hydroxy-nor-Arginine (nor-NOHA) (Sigma) was used at 5mM. The arginase inhibitor was added during stimulation with cl-CD95L and the again 24hrs later during *T. gondii* infection.

For the inhibition of cl-CD95L, mFas-Fc Chimera (R&D Systems Cat No. 435-FA) was added at 200ng/mL. The cl-CD95L inhibitor was added during stimulation with cl-CD95L and then again 24hrs later during *T. gondii* infection. For the inhibition of STAT6 (Sigma) was used at 0.2 mg/mL. The STAT6 inhibitor was added during stimulation with cl-CD95L and the again 24hrs later during *T. gondii* infection.

2.6. Cytokine assays- Mouse IL-1 beta and IL-6 Uncoated ELISA

Cytokine ELISAs for IL-6, IL-1β were conducted using commercially available kits from Invitrogen (Cat no. 88-7064-88, 88-7013, respectively).

In brief, the coating antibody was diluted adding 48µL of the antibody in 12mLs of coating buffer. 100µL of diluted coating antibody was then added to a 96 well ELISA plate (Corning Costar 9018) then left overnight at 4^{O}C. Thereafter, plates were washed 3 times between each step. Plates were blocked with 200µL of ELISA Diluent at room temperature for 1 hour. A 2-fold serial dilution was performed for the top standard to make the standard curve for a total of 8 points. 100µL of the samples were added to each well and 100µL of ELISA Diluent was then added to the blank well then left to incubate for two hours at room temperature. After which, 100 µL of diluted Detection Antibody was added to all wells. Plates were then sealed and incubated at room temperature for 1 hour. After the incubation, 100 µL of diluted Avidin-HRP was then added to each well for 30 minutes at room temperature. 1X TMB Solution was then added at 100 µL/well and incubated at room temperature for 15 minutes. Finally, 50 µL of Acid Stop Solution was added to all wells. The plate was then read at 450nm and 570nm. The values of 570nm were subtracted from those of 450nm for data analysis.

2.7. CellTiter-Glo® Luminescent Cell Viability Assay

The frozen supernatants from the VERO cell experiments were used for CellTiter-Glo® assays for the detection of ATP. 100 µL of each sample and media controls were added to a 96-well opaque plate. The plate was then left at room temperature for 30 minutes to equilibrate the plate. Thereafter, 100 µL of CellTiter-Glo® Reagent was added to each well. The contents were then mixed for 2 minutes on an orbital shaker. Finally, the plate was left to incubate at room temperature for 10 minutes to stabilise luminescent signal. Luminescence was then recorded using GloMax® luminometer.

2.8. LDH-Cytotoxicity Colorimetric Assay

The frozen supernatants from the VERO cell experiments are used for Cytotoxicity assays for the detection of LDH. This began with transferring 10 µL/well of each sample into of a clear 96-well plate (Corning Costar 9018). Thereafter, add 100 µL of the LDH Reaction Mix into each well and mix contents before incubating for 30 minutes at room temperature. Finally, measure the absorbance of the samples with a plate reader at 450 and 650 nm.

2.9. Arginase assay

The cell lysates from the BMDM infections are first thawed and centrifuged for 2 minutes at max speed to remove cells. 50µL of 10mM MnCl2/50mM Tris-HCl buffer (pH 7.5) and 50µL of lysate were added to an Eppendorf. After being mixed thoroughly the samples were incubated for 10 minutes at 55^{o}C for enzyme activation. After which, 50 µL of 0.5M L-arginine substrate (pH 9.7) was added to the activated lysate and incubated for 1 hour at 37^{o}C. To stop the reaction the acid-stop solution that was comprised of H_2SO_4 (96%), H_3PO_4 (85%), and H_2O in a ratio of 1:3:7 was added to the samples. To each standard, 600uL of acid stop solution was added whereas 400µL was added to each sample. Finally 9% isonitrosopriopherone was added to develop colour. To each standard, 37.5 µL of isonitrosopriopherone was added and then 25µL was added to each sample. After which, all standards and samples were heated to 103°C for 45 minutes and allowed to cool in darkness for another 10 minutes. 200 µL aliquots are then added to a 96 well ELISA plate (Corning Costar 9018) and the plate is measured at 540nm using plate reader.

2.10. BMDM preparation for Phospho-Kinase Array

BMDM were either left unstimulated or stimulated with 100ng/mL of cl-CD95L for 24 hours, after which BMDM were rinsed with PBS. BMDM were then solubilised at $1x10^7$ cells/mL in Lysis Buffer 6 (R&D systems). Samples were pipetted up and down in order to resuspended BMDM and then rock the lysate for 30 minutes at 2-8^oC. Samples were then centrifuged at 14,000 x g for 5 minutes and then transferred to an Eppendorf tube.

2.11. BCA Protein Assay

Firstly, 10µL of each sample was pipetted into a 96 well plate (Corning Costar 9018). 200µL of working reagent (Thermo scientific, Product No. 23227) was added to each well. Then standards were prepared and plate 10µL of each standard with 200µL of working reagent. The plate was then mixed thoroughly by being placed on a plate shaker for 30 minutes. Following this the plate was covered and incubated for 30 minutes at 37^oC. Once the plate has cooled to room temperature the absorbance was measured 560nm on a plate reader. A standard curve was prepared to determine the concentration of proteins in each unknown sample.

2.12. Phospho-Kinase Array

BMDM cell lysates were kept on ice prior to use. To begin protective sheets were removed from all membranes (Proteome Profiler, Phospho-Kinase Array, R&D Systems, Cat No. ARY003B). Each membrane was divided into two parts (A and B) and there was a total of 4 membranes. Each wells of an 8-Well Multi-dish was filled with 1.0mL of Assay Buffer 1, which served as a blocking buffer. After which, Part A and Part B were separated into the 8-Well Multi-dish and incubated on a rocking platform shaker for 1 hour. After incubation, the Assay Buffer 1 was aspirated and 1.0mL of the prepared samples were added to both parts A and B of each membrane. The plate was then incubated on a rocking platform shaker overnight at 2-8^{o}C. During each wash step membranes were carefully removed and placed in a plastic wash container with 20mLs of Wash Buffer. At this step Parts A and B were washed together. Each set of membranes were washed on a rocking platform shaker for 10 minutes and repeated for a total of 3 washes. The 8-Well Multi–dish was washed thoroughly with deionised water and dried. For all wells corresponding to Part A, 1.0mL of reconstituted Detection Antibody Cocktail A was added into Assay Buffer 1/ 2. For all Part B wells 1.0mL of reconstituted Detection Antibody Cocktail B was added into Assay Buffer 1/ 2. After, allowing excess Wash Buffer to drain from each membrane they were placed into appropriate wells. Then the plate was incubated on rocking platform shaker for 1 hour at room temperature. After incubation, the membranes were washed as described above however Part A and B were washed in separate containers. As the membranes were being washed, 1.0 mL of diluted

Streptavidin-HRP was added into Array Buffer 2/ 3 and then into each of the wells of the plate. Once again excess wash buffer was removed from membranes and they were placed back in corresponding well. Plates were incubated on a rocking platform shaker for 30 minutes at room temperature. After incubation once again, membranes and plate were washed as described above where Part A and B were washed in the same container. Each membrane was carefully removed, and the edge was blotted on a paper towel to drain excess Wash Buffer. Then each membrane was placed on a plastic sheet with its corresponding Part A and Part B end-to-end. After which, 1.0mL of Chemi Reagent Mix was added to each membrane set and incubated for 1 minute. After incubation the excess Chemi Reagent Mix was removed from each membrane. Then membranes were placed facing upwards on an autoradiography film cassette and exposed to X-ray film. Results were then analysed using ImageJ software to detect Pixel Density. Based on average pixel densities a heat map was created to show all upregulated pathways.

2.13. Statistics

Raw data was collected on Microsoft Excel workbooks. All data analysis was conducted using Prism 7 (GraphPad). Unpaired T-test was used to compare the means of two unmatched groups, assuming that the values follow a Gaussian distribution. Due to the data obtained during the study being parametric it was decided that an Unpaired T-test was the most suitable statistical method for comparing parasite counts during this study. A Two-way ANOVA determines how a response is affected by two factors. For this reason, a Two-way ANOVA was used to test the parametric data collected from all ELISA and Arginase assays; as parasite and cl-CD95L were two independent factors. A Tukey multiple comparison test was used as a post-test to compare every mean with every other mean. P values of <0.05 were taken as significant.

2.14. Ethics

All mice were culled by cervical dislocation schedule 1 procedure and tissue transported on ice to IC2 (Liverpool Science Park) where experiments were carried out. All mice were maintained in accordance to the Animal Scientific Procedures Act 1986 (ASPA). Tissues were obtained from excess breeding stocks maintained as part of a GM mouse colony and were collected as breeding stock animals were removed from the colony.

Chapter 3. Results

To initially determine if cl-CD95L had an effect on *T. gondii*-host dynamics we tested the effects of cl-CD95L in well characterised VERO cells.

3.1. cl-CD95L exacerbates *T. gondii's* destructive capabilities in VERO cells

We began by demonstrating the effect of pre-exposure to cl-CD95L on monolayer destruction in VERO cells following *T. gondii* infection. The level of VERO cell monolayer destruction was measured visually. Figure 4 shows the reference images to illustrate how the grades of monolayer destruction were measured across treatments. The data collected from these monolayer destruction experiments is shown in Figure 5. From this data it can be seen that after infection with *T. gondii*, the percentage of destruction increases from 2% in uninfected/unstimulated to 14% in infected/unstimulated. Moreover, when cells are pre-exposed to cl-CD95L it was found that monolayer destruction was higher after *T. gondii* infection have higher levels when compared to unstimulated cells from 5% to 20% (P value <0.0001). Significant differences were also found between all uninfected and infected cells across when cells were stimulated with cl-CD95L in the range of 50, 100, and 200ng/mL (P value <0.0001). Finally, a significant difference was found between infected/unstimulated cells and with cells that were infected/stimulated with 100ng/mL of cl-CD95L (P value <0.05).

On the basis of these results we proceeded to use 100ng/mL of cl-CD95L in further experiments.

Figure 4. Reference images of different stages of VERO cell monolayer destruction (Zeiss Axiovert 25, X10 magnification)
A) Shows VERO cells with no monolayer destruction due to *T. gondii* infection
B) Shows VERO cells infected with *T. gondii*

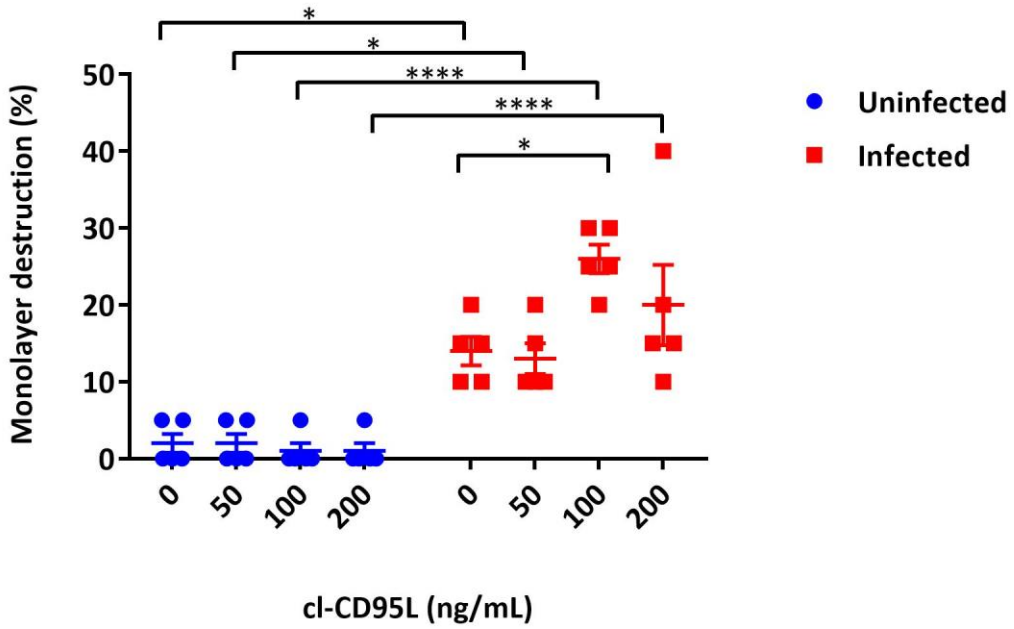

Figure 5. Percentage monolayer destruction intensifies after stimulation and infection

VERO cells were either stimulated with doses of cl-CD95L as indicated. After 24 hours the cells were then infected with *T. gondii* at a MOI of 5 or left uninfected. After a further 48 hours post infection the percentage of monolayer was estimated for each group by examination under a light microscope. Each treatment was performed in triplicate and data shown above presents 6 independent experiments. Data were analysed in GraphPad Prism using an Two way ANOVA to detect significant differences between stimulated and unstimulated cells compared to their infected counter parts (****P<0.0001). Significance was also found between in infected cell that were either unstimulated or stimulated with cl-CD95L at 100ng/mL (*P<0.05).

3.2. Tachyzoite replication is amplified with the addition of cl-CD95L

Having demonstrated a combined effect of *T. gondii* and cl-CD95L on monolayer destruction we next determined the effect of stimulation with cl-CD95L on *T. gondii* replication levels. VERO cells were stimulated with 100ng/mL of cl-CD95L and then infected with *T. gondii.* The number of parasites in the supernatant was determined after 48 hours of infection by manual counting using a haemocytometer. Figure 6 demonstrates that a significant increase in parasite replication occurs after stimulation ($12x10^4$/mL parasites) with cl-CD95L when compared to unstimulated ($9x10^4$/mL parasites) VERO cells. (P value <0.05).

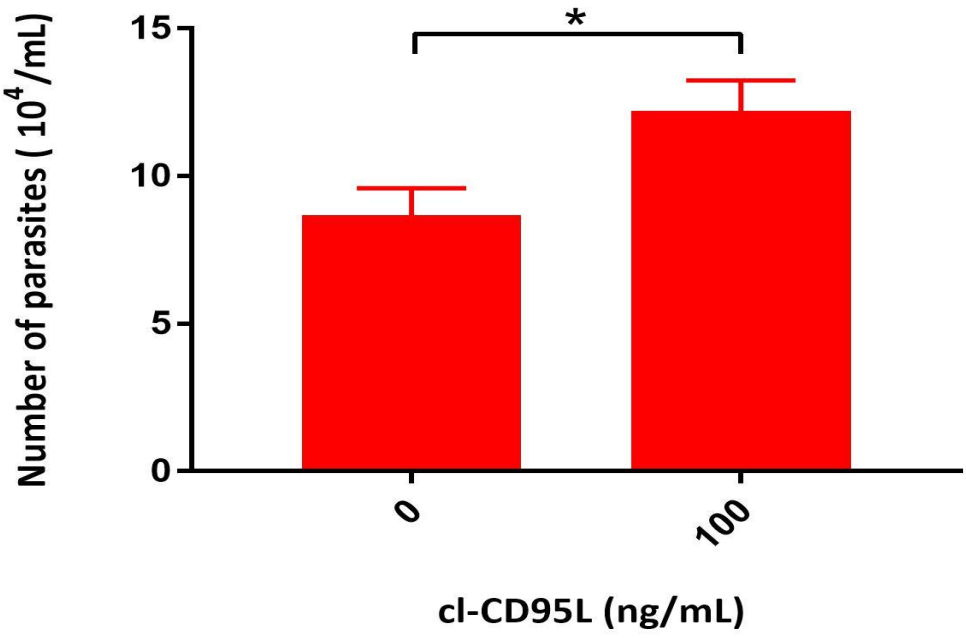

Figure 6. Free parasite counts with or without stimulation with cl-CD95L at 48hours post infection

VERO cells were either stimulated with 100ng/mL of cl-CD95L or left unstimulated. After 24 hours the cells were then infected with *T. gondii* at a MOI of 5 or left uninfected. After a further 48 hours post infection the parasite number was counted for each group using a haemocytometer under a light microscope. Each treatment was performed in triplicate and data shown above presents 5 independent experiments. Data were analysed in GraphPad Prism using an Unpaired T test to detect significant differences between stimulated and unstimulated cells which had been infected (*P <0.05).

3.3. cl-CD95L increases cell viability with limited impact on cytotoxicity

Parasite replication involves cell egress resulting in host cell destruction before infection of a new cell. Having demonstrated that the increase in monolayer destruction above might be caused by the increase in *T. gondii* replication because of cl-CD95L stimulation we sought to determine if cl-CD95L was causing these effects by an increase in cytotoxicity or by making host cells permissive. To test for increases in cytotoxicity we first measured ATP in the supernatants of cultures as a proxy for replicating host cells.

These results demonstrate that before infection, as expected, there are high levels of cell viability (Figure 7 A). It can also be seen that there is a significant increase in cell viability in uninfected/stimulated cells when compared to uninfected/unstimulated (P value <0.05). In comparison, after infection with *T. gondii* the levels of cell viability, both unstimulated and stimulated with cl-CD95L, was reduced. In addition to this, VERO cells stimulated with 100ng/mL of cl-CD95L had a significant increase in cell viability under unstimulated/infected conditions (P value <0.001).

To evaluate if cl-CD95L was altering the cytotoxicity of *T. gondii* during VERO cell infection we measured lactate dehydrogenase (LDH) in the supernatants. The results show a significant difference between cytotoxicity levels of uninfected and infected cells after stimulation to cl-CD95L (P value <0.001). A Significant difference was also found between uninfected/stimulated cells and cells which had been infected/stimulated (P value <0.05).

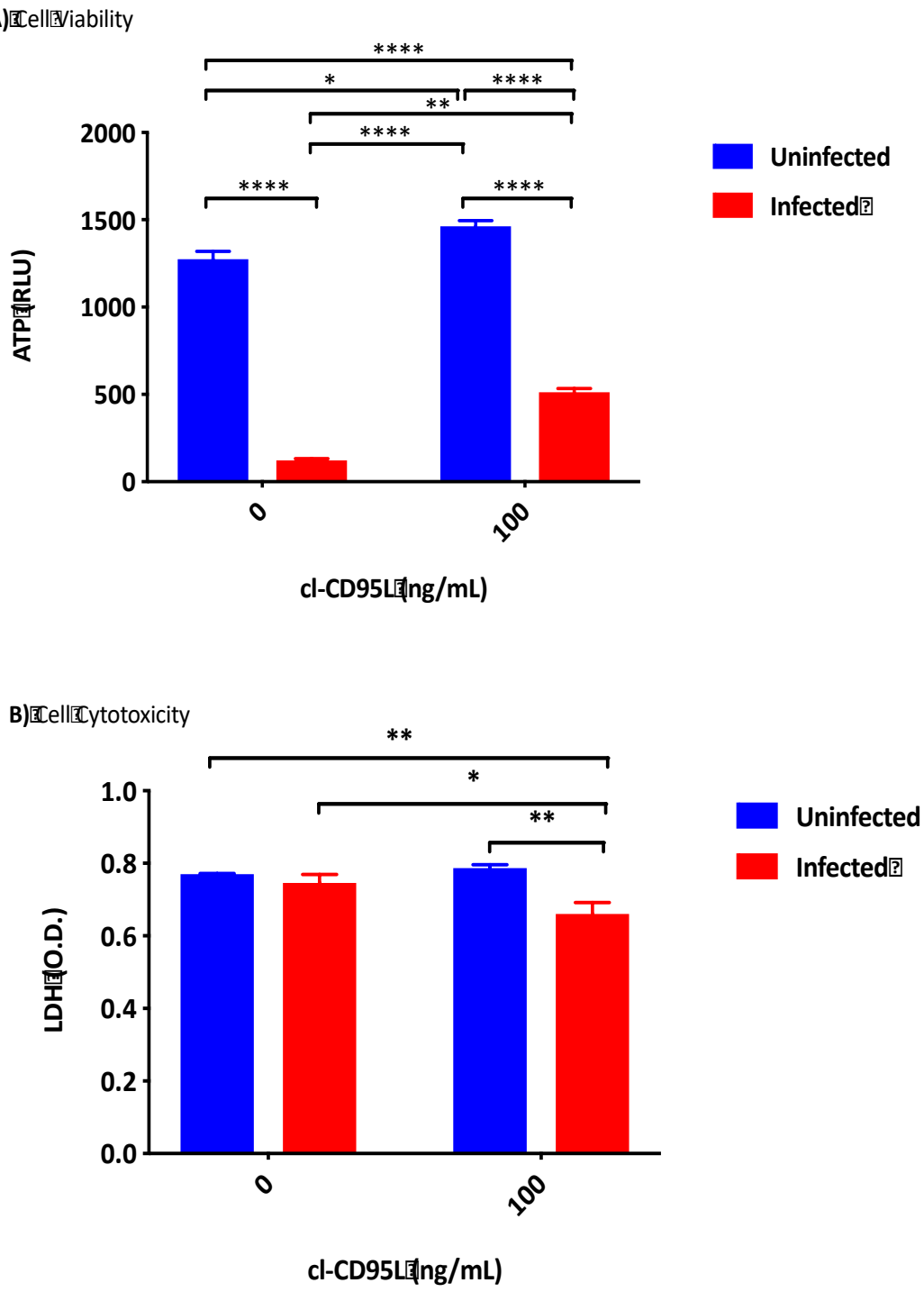

Figure 7. cl-CD95L does alter viability and cytotoxicity prior to *T. gondii* infection

VERO cells were either stimulated with 100ng/mL of cl-CD95L or left unstimulated. After 24 hours the cells were then infected with *T. gondii* at a MOI of 5 or left uninfected. After 48 hours the supernatant were analysis. Each treatment was performed in triplicate and data shown above presents 5 independent experiments. Data were analysed in GraphPad Prism using a 2 way ANOVA. **A)** A Cell Titer-Glo assay was used to determine cell viability. Significant differences were found between stimulated and unstimulated cells, which have not been infected (*P=0.05). Significant difference was also found between stimulated and unstimulated cells which had been infected (**P<0.001).

Significant difference was found between uninfected cells that were unstimulated and their infected counter part (****P<0.0001). The same was found between infected cells that were stimulated with 100ng/mL of cl-CD95L and their infected counterpart (****P<0.0001). **B)** A LDH assay was used to determine the levels of cytotoxicity. Significant differences were found between stimulated and unstimulated cells, which had been infected (*P<0.05). Significant difference was also found between stimulated and uninfected cells and cells, which had been stimulated and infected (**P<0.05). Significant difference was also found between uninfected cells that were unstimulated and stimulated cells which had been infected (**P<0.001).

3.4. cl-CD95L alters BMDM responses during infection with *T. gondii*

After demonstrating a change in response of VERO cells due to cl-CD95L exposure we wanted to further investigate if these responses were also present in BMDM and if there was a particular change in the immune response associated with it. As an initial experiment we investigated the impact of stimulation with cl-CD95L on parasite replication in BMDM by preforming parasite counts after 48 hours of infection.

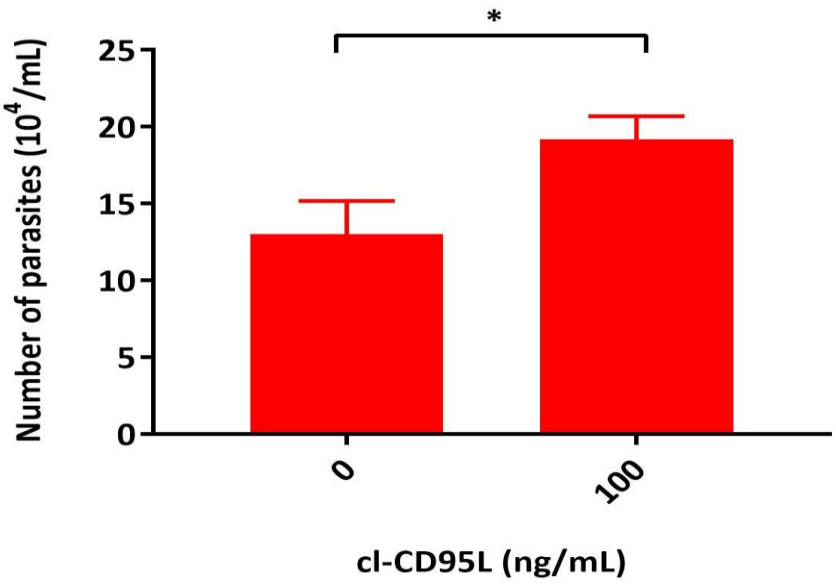

Figure 8. Free parasite counts from BMDM stimulated with cl-CD95L and infected with *T. gondii*

BMDM were either stimulated with 100ng/mL of cl-CD95L or left unstimulated. After 24 hours the BMDM (10^5 per well) were then infected with *T. gondii* at a MOI of 5 or left uninfected. After a further 48 hours post infection the parasite number was counted for each group using a haemocytometer under a light microscope. Each treatment was performed in triplicate and data shown above presents 5 independent experiments. Data were analysed in GraphPad Prism using an Unpaired T test to detect significant differences between stimulated and unstimulated cells which had been infected (*P<0.05).

BMDM were then used to determine if a cytokine response could be elicited following exposure to *T. gondii* and if differing doses of cl-CD95L could alter this. ELISAs were used to detect levels of IL-1 β and IL-6 from the supernatant. These were then used to determine the expected responses during stimulation and infection of BMDM to compare the inhibition of different pathways.

To determine the effect of stimulation with cl-CD95L on *T. gondii* replication levels, BMDM cultures were stimulated with 100ng/mL of cl-CD95L and then infected with *T. gondii*. The number of parasites in the supernatant was determined after 48 hours of infection by counting using a haemocytometer. Figure 8 shows a significant increase in parasite counts after BMDM cultures have been stimulated with cl-CD95L when compared to unstimulated (P value <0.05). Using an ELISA kit the supernatants were analysed for the presence of IL-1 β. The data in Figure 9. A) shows that the production of IL-1β increases significantly by BMDM after infection using *T. gondii* tachyzoites. After using a 2-way ANOVA it has been shown that there was no significant difference between unstimulated and stimulated with cl-CD95L doses between 50-200ng/mL when comparing uninfected data, the same was true when comparing infected data. However, a significant difference was found when comparing uninfected and infected data (P value <0.0001).

The presence of IL-6 was also evaluated from the BMDM supernatants to determine the effect of cl-CD95L before and after infection with *T. gondii*. The data in Figure 9. B) illustrates that levels of IL-6 secretion by BMDM is significantly higher in BMDM that have been infected with *T. gondii* (P value <0.0001). Once infected, both unstimulated and stimulated BMDM produce increased levels of IL-6. After using a 2-way ANOVA it has been shown that there was significant difference between unstimulated and stimulated with cl-CD95L at 100ng/mL (P value <0.05).

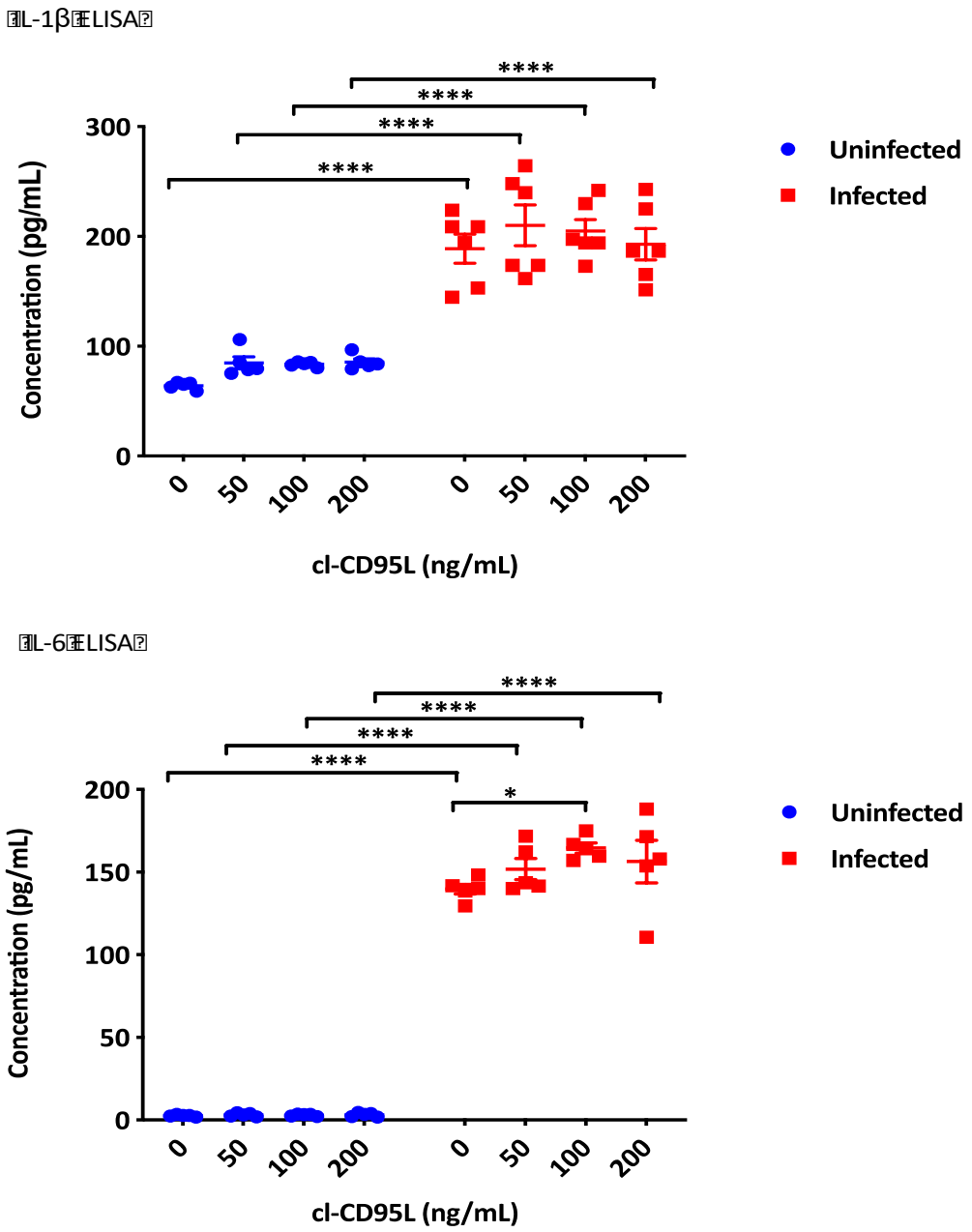

Figure 9. *T. gondii* and cl-CD95L synergistically alter cytokine responses.

BMDM were stimulated using indicated doses of cl-CD95L. After 24 hours the BMDM (10^5 per well) were then infected with *T. gondii* at a MOI of 5 or left uninfected. After a further 48 hours supernatants were assayed for IL-1β and IL-6 using an ELISA kit. Each treatment was performed in triplicate and data shown above presents 5 independent experiments. Data were analysed in GraphPad Prism using a 2 way ANOVA to detect significant differences. **A) IL-1β-** Significant differences were found between BMDM which had been either stimulated or unstimulated and compared to their infected counterparts (****P<0.0001). **B) IL-6-** Significant differences were found between BMDM which had been either stimulated or unstimulated and compared to their infected counterparts (****P<0.0001). A significant difference was also found between infected BMDM, which had been unstimulated and stimulated with 100ng/mL of cl-CD95L (*P<0.05).

The impact of cl-CD95L and *T. gondii* tachyzoites on the arginase activity of BMDM was determined using an arginase assay. The cell lysate was used to demonstrate arginase activity changes. Figure 10 shows that the arginase activity increases significantly after BMDM have been infected with *T. gondii* with both unstimulated and stimulated conditions (P value <0.0001). Additionally, with BMDM stimulation with cl-CD95L and 48hrs post infection the level of arginase activity was significantly higher than its unstimulated counterpart (P value <0.0001).

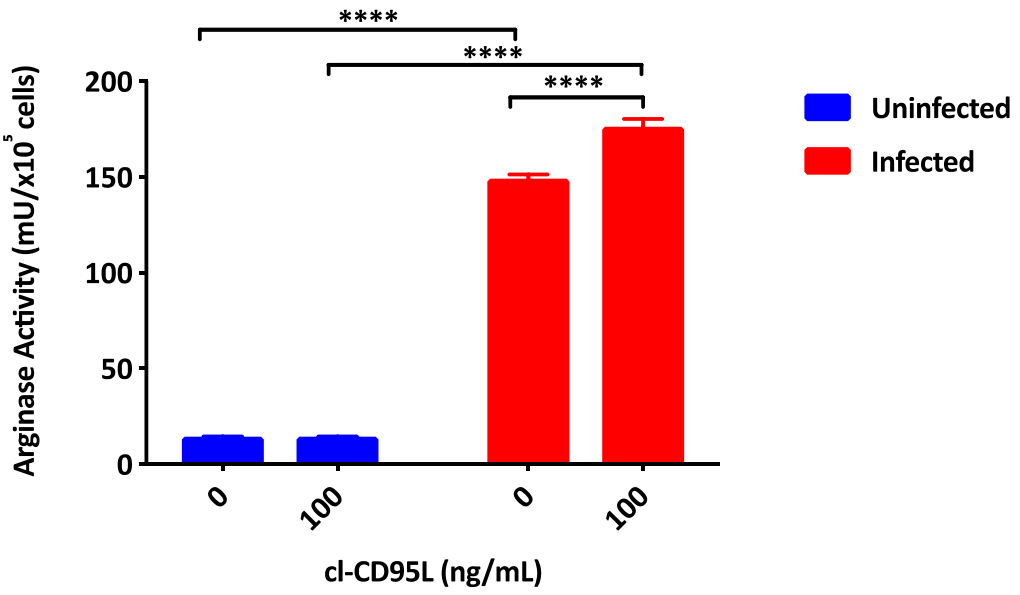

Figure 10. Arginase assay using cl-CD95L at 100ng/mL after 48hours post infection.

BMDM were stimulated using cl-CD95L at 100ng/mL. After 24 hours the BMDM (10^5 per well) were then infected with *T. gondii* at a MOI of 5 or left uninfected. After a further 48 hours cell lysates were assayed using an arginase assay. Each treatment was performed in triplicate and data shown above presents 6 independent experiments. Data were analysed in GraphPad Prism using a 2-way ANOVA to detect significant differences between stimulated and unstimulated cells which had been infected (****P<0.0001) Significance was also found between uninfected and infected BMDM in both unstimulated and stimulated conditions (****P<0.0001).

3.5. The impact of inhibiting cl-CD95L during a *T. gondii* infection

To better understand the effect cl-CD95L has on BMDM during a *T. gondii* infection we inhibited the ligand and compared the inhibited response to that of a normal BMDM-cl-CD95L response. Figure 11. A) demonstrates that the inhibition of cl-CD95L causes a significant difference in the production of IL-6 by BMDM after infection with *T. gondii* when compared to expected value of infected BMDM stimulated with 100ng/mL (P value <0.05). As expected, there was a significant difference in IL-6 production in uninfected BMDM, which had not been stimulated with cl-CD95L when compared to their infected counterpart (P value <0.0001). Similarly, this significant difference was also found in uninfected BMDM, which have been stimulated with 100ng/mL of cl-CD95L when compared to their infected counterpart (P value <0.0001).

To evaluate the impact of inhibiting cl-CD95L on BMDM arginase activity during a *T. gondii* infection a cl-CD95L inhibitor was added during stimulation and then again when infected with *T. gondii* tachyzoites. The data in Figure 11. B) suggests that the inhibition of cl-CD95L causes a significant difference in arginase activity in stimulated BMDM when compared to expected stimulated values (P value <0.001). As expected in the vehicle control BMDM, there is a significant decrease in arginase activity in unstimulated BMDM when compared to stimulated BMDM (P value <0.05).

To demonstrate the effect of inhibiting cl-CD95L on tachyzoite replication in BMDM, parasite counts were taken at 48 hours post infection using a haemocytometer. From the results in Figure 11. C) we can show that there is a significant decrease in parasite numbers when cl-CD95L is inhibited when compared to expected parasite numbers in stimulated BMDM (P value <0.05). As expected in the control BMDM cultures a significant increase in parasite counts is seen in infected/stimulated BMDM when compared to infected/unstimulated BMDM (P value <0.05).

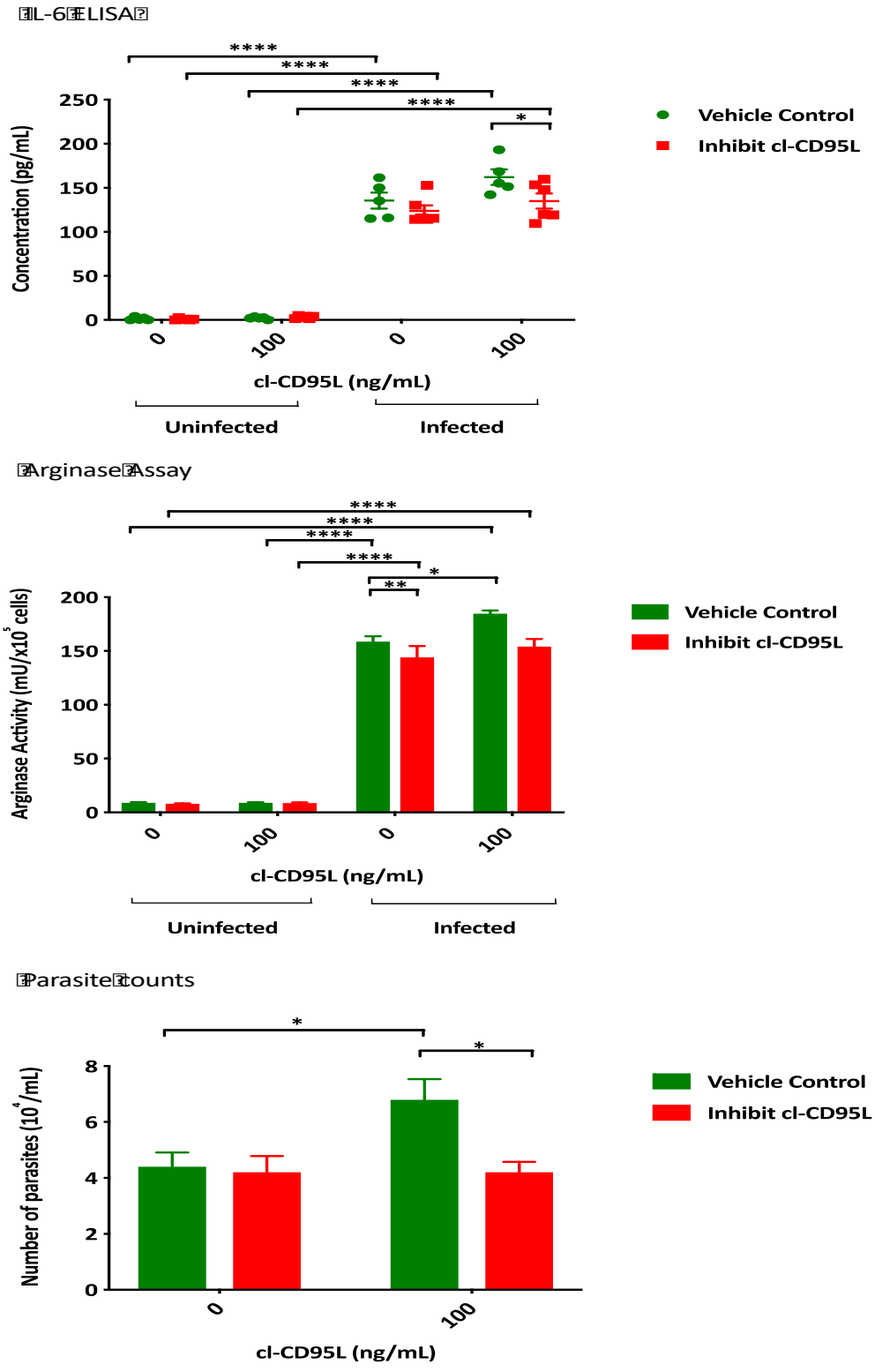

Figure 11. The impact of a cl-CD95L inhibitor on BMDM function. A) IL-6 ELISA using a cl-CD95L inhibitor then stimulating with cl-CD95L dose stimulations at 48hours post infection B) Arginase assay using cl-CD95L inhibitor with cl-CD95L doses after 48hours post infection C) Free parasite counts using cl-CD95L inhibitor on BMDM stimulated with cl-CD95L and infected with *T. gondii*

BMDM had cl-CD95L inhibited and were then stimulated using indicated doses of cl-CD95L. After 24 hours the BMDM (10^5 per well) were then infected with *T. gondii* at a MOI of 5 or left uninfected. After a following 48 hours samples were analysed. Each treatment was performed in triplicate and data shown above presents 6 independent experiments for inhibited samples and a further 5 independent experiments for control samples. Data were analysed in GraphPad Prism using a 2-way ANOVA to detect a significant difference**. A)** The supernatants were analysed using an IL-6 ELISA Kit. Significant differences were found between infected and stimulated BMDM, which have a cl-CD95L inhibitor when compared to the expected values (*P<0.05). Expected values also show significant difference when comparing uninfected BMDM which have either been left unstimulated or stimulates with 100ng/mL of cl-CD95L when compared to infected counterparts (****P<0.0001). **B)** The cell lysates were analysed using an arginase assay. Significant differences were found between stimulated BMDM pre-exposed to a cl-CD95L inhibitor when compared to stimulated BMDM with no inhibitor (**P<0.001). Significance is also seen between unstimulated and stimulated BMDM (*P<0.05). **C)** By preforming parasite counts, significant differences were found between stimulated BMDM, which have been pre-exposed to a cl-CD95L inhibitor when compared to expected values from non-inhibited BMDM (*P<0.05). A significant difference was also found between unstimulated and stimulated BMDM (*P<0.05).

3.6. The inhibition of STAT6 thwarts BMDM responses

To determine the role of STAT6 during stimulation with cl-CD95L and parasite infections with *T. gondii* we inhibited STAT6 and compared the results to the expected values. Initially the supernatant from BMDM, which had been pre-exposed to a STAT6 inhibitor, were analysed for the presence of IL-6. Figure 12. A) demonstrates how after STAT6 has been inhibited the level of IL-6 secretion by BMDM that have been stimulated with cl-CD95L decreases significantly when compared to the expected values (P value <0.001). This is also true in BMDM that are left unstimulated but pre-exposed to a STAT6 inhibitor when compared to expected values (P value <0.0001). As expected, there is a significant decrease in IL-6 production found between uninfected BMDM when compared to their infected counterparts, this is independent of stimulation with cl-CD95L (P value <0.0001).

To evaluate the impact of inhibiting STAT6 on BMDM arginase activity during a *T. gondii* infection a STAT6 inhibitor was added during stimulation and then again when infected with *T. gondii* tachyzoites. The data in Figure 14. B) suggests that the inhibition of STAT6 leads to a significant reduction in arginase activity after infection with *T. gondii.* We found that when uninfected the level of arginase reduction in STAT6 inhibited BMDM was significant in both unstimulated and stimulated when compared to expected values (P value <0.05 for both unstimulated and stimulated). However, once infected the significant reduction in arginase activity is greater between STAT6 inhibited BMDM in both

unstimulated and stimulated counterparts when compared to expected values (P value <0.0001). As expected with non-inhibited BMDM, arginase activity increases after infection independent of stimulation with 100ng/mL of cl-CD95L (P value <0.0001).

To determine the impact of inhibiting STAT6 on parasite replication in BMDM, parasite counts were taken at 48 hours post infection using a haemocytometer. This impact is illustrated in Figure 14 C), showing that when stimulated BMDM are pre-exposed to a STAT6 inhibitor the number of parasites decreases significantly when compared to expected values (P value <0.001). As expected, there is a significant increase in parasite numbers after BMDM have been stimulated with 100ng/mL of cl-CD95L when compared to unstimulated BMDM (P value <0.05).

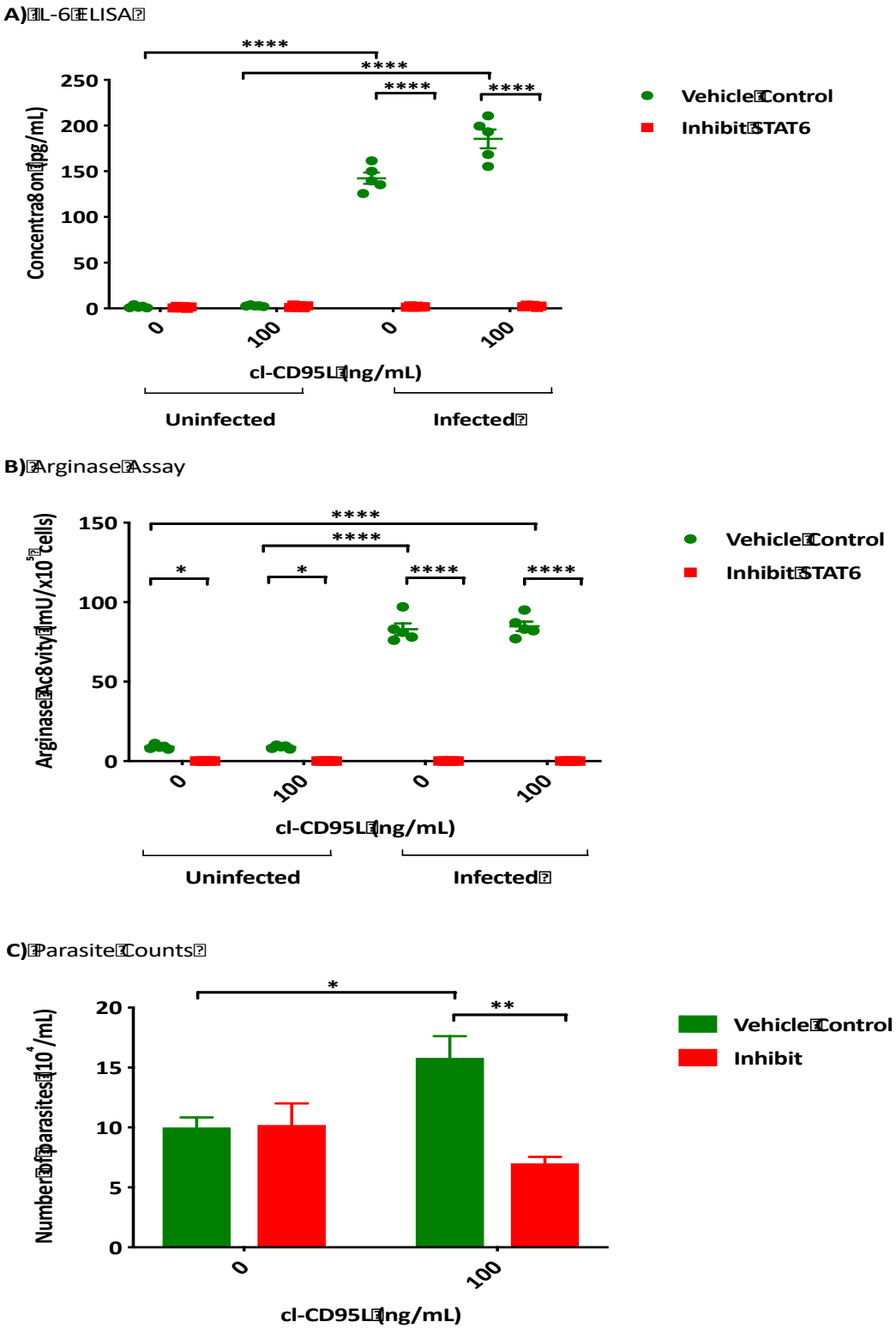

Figure 12. The inhibition of STAT6 shows the mechanical role of STAT6 in BMDM. A) IL-6 ELISA using a STAT6 inhibitor then stimulating with 100ng/mL of cl-CD95L at 48hours post infection B) Arginase assay using STAT6 inhibitor with 100ng/mL of cl-CD95L after 48hours post infection C) Free parasite counts using STAT6 inhibitor on BMDM stimulated with cl-CD95L and infected with *T. gondii.*

BMDM had arginase inhibited and were then stimulated using 100ng/mL of cl-CD95L. After 24 hours the BMDM (10^5 per well) were then infected with *T. gondii* at a MOI of 5 or left uninfected. After a following 48 hours samples were analysed. Each treatment was performed in triplicate and data shown above presents 6 independent experiments for inhibited samples and a further 5 independent experiments for control samples. Data were analysed in GraphPad Prism using a Two-way ANOVA to detect significant differences. **A)** The supernatant was analysed using an IL-6 ELISA Kit. Significant differences were found between infected BMDM pre-exposed to a STAT6 inhibitor that have been stimulated or unstimulated when compared to their expected values (****P<0.0001). Significant differences have also been shown between uninfected BMDM that have been left unstimulated or stimulated when compared to their infected counterparts (****P<0.0001). **B)** The cell lysate was analysed using an arginase assay. Significant differences were found between stimulated and unstimulated BMDM, which have been pre-exposed to a STAT6 inhibitor when compared to expected values (*P<0.05/ ****P<0.0001). A significant difference has been found between uninfected BMDM and their infected counter parts (****P<0.0001). C) By preforming parasite counts, significant differences were found between stimulated BMDM which have been infected compared to stimulated and STAT6 inhibited (**P<0.001). A significant difference has also been found between parasite numbers from unstimulated and stimulated BMDM (*P<0.05).

3.7. IL-6 secretion influences BMDM responses during infection

Before investigating the effects that neutralising IL-6 has on *T. gondii* infections we first had to ensure the IL-6 neutralising antibody worked effectively. By carrying out an IL-6 EILSA using normal conditions as the control we were able to demonstrate the effectiveness of the neutralising antibody. Figure 13. A) illustrates that the anti-mouse IL-6 neutralising antibody (120ng/mL) works effectively by showing no levels of IL-6 detected from the samples. Significant differences were found between all infected BMDM with doses of cl-CD95L and left unstimulated when comparing normal conditions and when IL-6 is neutralised (P value <0.0001).

To demonstrate the effect neutralising IL-6 has on the arginase activity of BMDM an IL-6 neutralising antibody was added to samples and compared to the expected values from an arginase assay. Figure 12. B) shows IL-6 neutralisation and infected BMDM that have been stimulated with 100ng/mL of cl-CD95L have significantly lover arginase activity in comparison to stimulated BMDM that are infected with no neutralising antibody added, which have been used as a control (P value <0.0001). This decrease in arginase activity can also be seen with infected unstimulated BMDM with anti-IL-6 when compared to their non-neutralised counter-part (P value <0.001). BMDM cultures display higher arginase activity when infected in comparison to their uninfected counterparts, this is independent of stimulation with cl-CD95L and IL-6 neutralisation (P value <0.0001).

In order to investigate how parasite replication is affected by the neutralisation of IL-6 secretion by BMDM parasite counts were taken at 48hrs post *T. gondii* infection. To determine the effect of stimulation with cl-CD95L has on *T. gondii* levels replication, BMDM were stimulated with 100ng/mL of cl-CD95L and then infected with *T. gondii*. The number of parasites in the supernatant was determined after 48 hours of infection by counting using a haemocytometer. Shown in Figure 12. C) are parasite counts taken from the IL-6 neutralised BMDM that have been stimulated with 100ng/mL of cl-CD95L and compared to expected parasite numbers. From this it is clear that when IL-6 is neutralised in stimulated BMDM parasite numbers decrease significantly (P value <0.0001). However, there was no significance found between unstimulated BMDM when IL-6 was neutralised when compared to expected values. As expected in the vehicle control BMDM, there is a significant increase in parasite numbers when BMDM are stimulated with cl-CD95L when compared to unstimulated BMDM (P value <0.05).

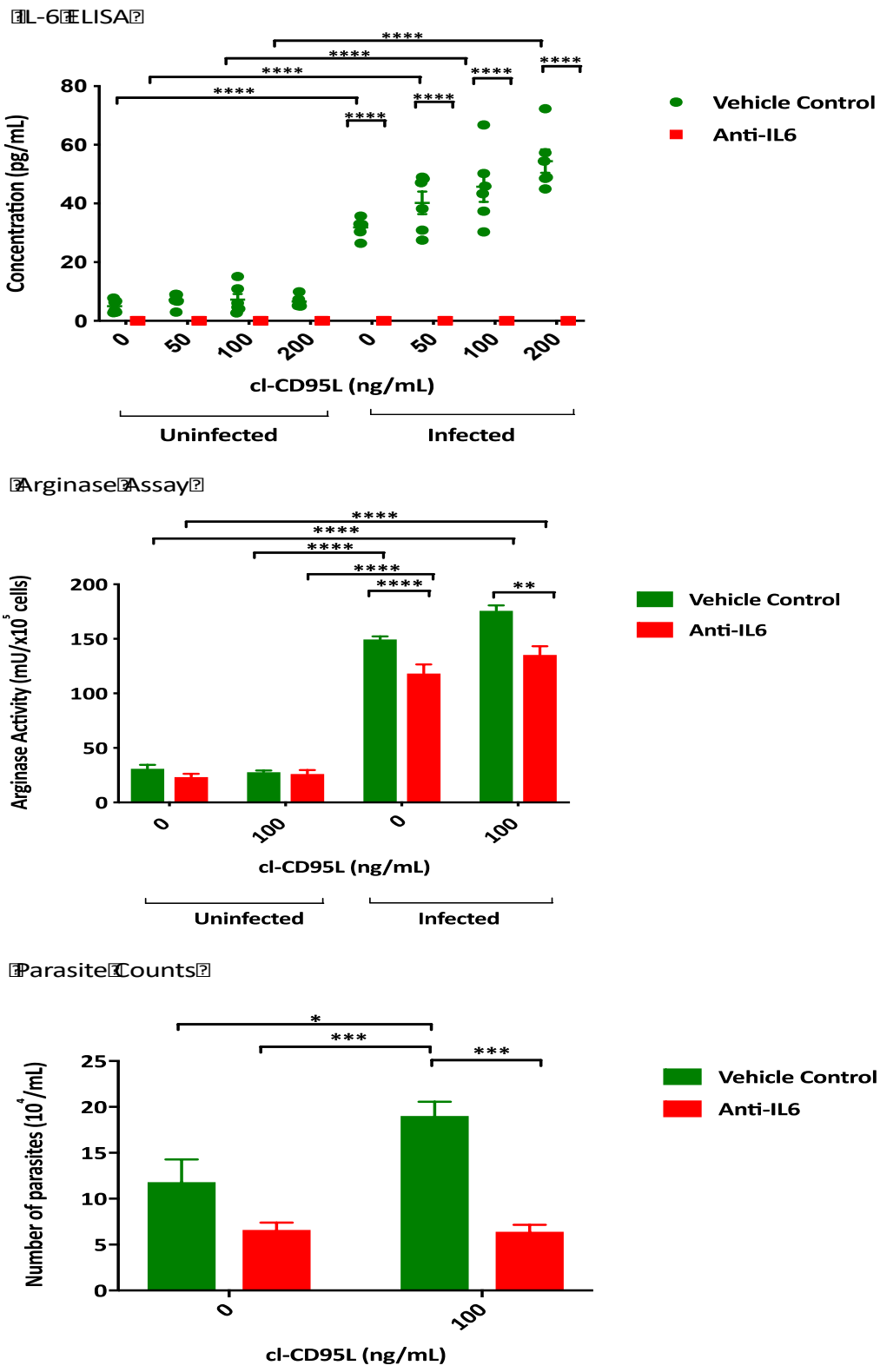

Figure 13. Anti-IL6 demonstrates IL-6 as a critical cytokine in BMDM functioning. A) IL-6 ELISA using anti-IL6 with cl-CD95L dose stimulations at 48hours post infection B) Arginase assay using anti-IL6 with cl-CD95L doses after 48hours post infection C) Free parasite counts using anti-IL6 on BMDM stimulated with cl-CD95L and infected with *T. gondii.*

BMDM were stimulated using indicated doses of cl-CD95L. After 24 hours the BMDM (10^5 per well) were then infected with *T. gondii* at a MOI of 5 or left uninfected. Anti-IL6 was added during stimulation with cl-CD95L and again during infection. After a further 48hrs samples were analysed. Each treatment was performed in triplicate and data shown above presents 6 independent experiments. Data were analysed in GraphPad Prism using a 2-way ANOVA to detect significant differences **A)** The supernatants were analysed using an IL-6 ELISA Kit significant differences were found between BMDM infected with T. gondii with does of cl-CD95L and left unstimulated when comparing control and when IL-6 is inhibited (****P<0.0001). **B)** The cell lysates were analysed using an arginase assay a significant difference was found between infected BMDM with anti-IL6 which have been stimulated with 100ng/mL cl-CD95L when compared to non-inhibited infected BMDM which has also been stimulated with 100ng/mL of cl-CD95L (****P<0.0001). A significant difference has been found between infected BMDM with anti-IL-6 without stimulation when compared to their non-inhibited counterparts (**P<0.001). Significant difference has also been found between uninfected BMDM when compared to infected BMDM independent of inhibitors and stimulation (****P<0.0001). **C)** By preforming parasite counts a significant differences was found between stimulated with no anti-IL-6 and stimulated with anti-IL-6 (***P<0.001). A significant difference was also found between stimulated and unstimulated BMDM (*P<0.05).

3.8. Arginase inhibition limits BMDM responses to *T. gondii*

Previously in Figure 10 it has been demonstrated that Arginase is significantly increased after infection with *T. gondii*, particularly after stimulation with cl-CD95L. In order to investigate the impact of arginase in relation to cl-CD95L and *T. gondii* BMDM were pre-exposed to an arginase inhibitor during stimulation and then again during infection.

Before investigating the impact of inhibiting arginase on *T. gondii* infections we first had to ensure the arginase inhibitor worked effectively. By carrying out an arginase assay using normal conditions as the control we were able to demonstrate the effectiveness of the inhibitor. Figure 13. A) illustrates that the arginase inhibitor did not work effectively as even with a reduced detection level of arginase in comparison to expected value, the levels of arginase activity are still high. However, there is a significant difference between infected and simulated BMDM pre-exposed to an arginase inhibitor when compared to vehicle control BMDM (P value <0.0001). This significant difference can also be seen between infected and unstimulated BMDM when comparing pre-exposed to an arginase inhibitor to the expected values (P value <0.0001). As expected in the vehicle control BMDM, uninfected cells show a significantly lower arginase activity when compared to infected BMDM, independent of stimulation with cl-CD95L (P value <0.0001). Following on from this, infected and stimulated BMDM display a significantly higher arginase activity when compared to unstimulated and infected BMDM (P value <0.0001).

The effect arginase has on IL-6 secretion by BMDM has been illustrated below using an IL-6 ELISA kit. From Figure 13. B) it has been shown that there is a significant difference between the levels of IL-6 produced by arginase inhibited BMDM that have been stimulated with 100ng/mL of cl-CD95L when compared with stimulated BMDM, which have not been inhibited (P value <0.05). As expected in the vehicle control BMDM, there is a significant difference in IL-6 production between uninfected and infected BMDM independent of stimulation with cl-CD95L and inhibitors (P value <0.0001).

To better understand the effect of inhibiting arginase on parasite replication in BMDM, parasite counts were taken at 48 hours post infection. Figure 13. C) illustrates that when arginase is inhibited in unstimulated BMDM, there is no significant difference in parasite numbers when compared to expected values. However, there is a significant decrease in parasite counts when arginase is inhibited in BMDM stimulated with 100ng/mL of cl-CD95L when compared to control values (P value <0.05). As expected in normal conditions there is also a significant increase in parasites numbers found in BMDM stimulated with 100ng/mL of cl-CD95L when compared to unstimulated BMDM (P value <0.05).

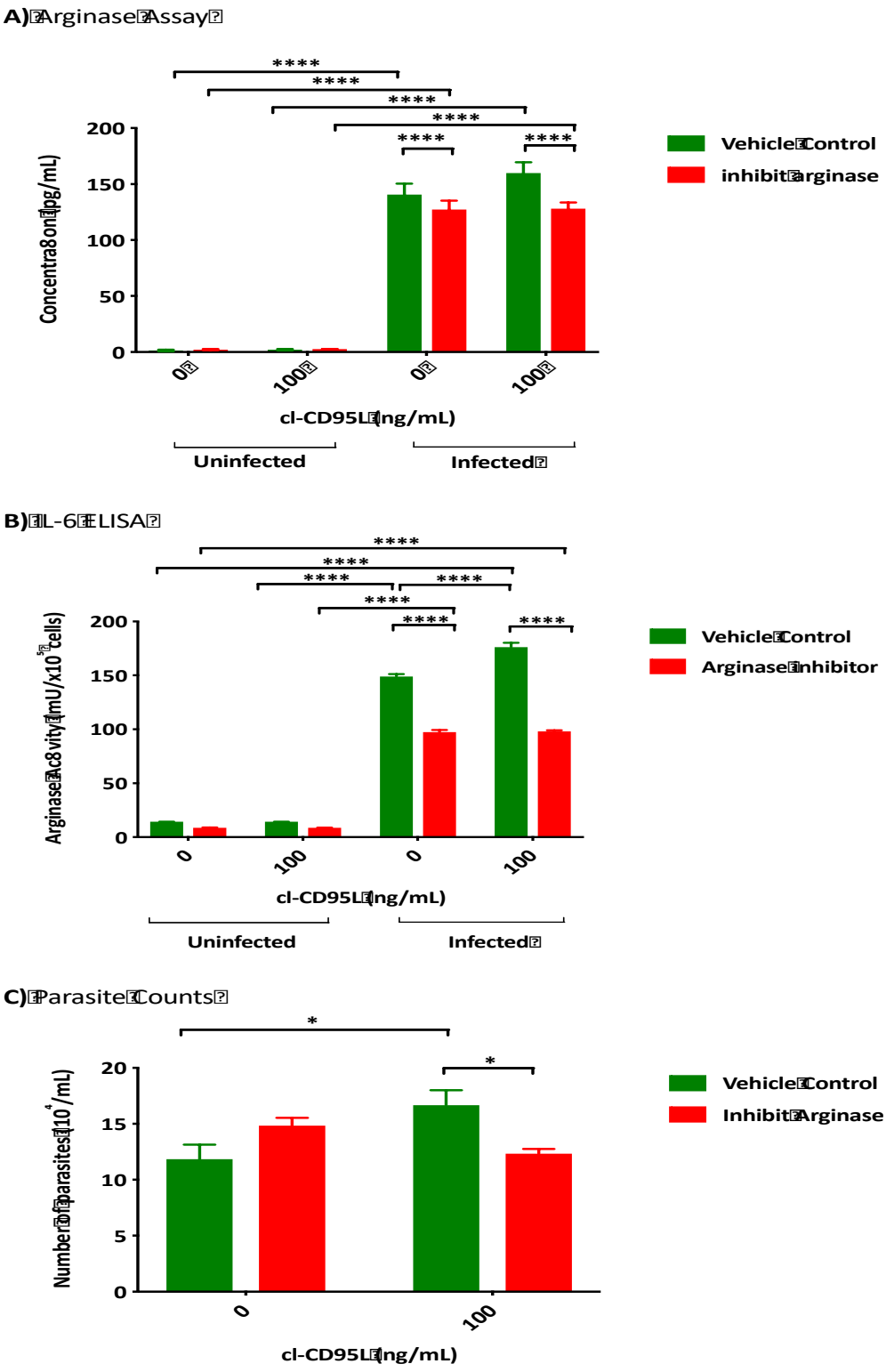

Figure 14. The inhibition of Arg-1 showing the role of Arg-1 in BMDM. A) Arginase assay using arginase inhibitor with 100ng/mL of cl-CD95L at 48hours post infection B) IL-6 ELISA using an arginase inhibitor then stimulating with 100ng/mL of cl-CD95L at 48hours post infection C) Free parasite counts using an arginase inhibitor on BMDM stimulated with cl-CD95L and infected with *T. gondii.*

BMDM were stimulated using 100ng/mL of cl-CD95L. After 24 hours the BMDM (10^5 per well) were then infected with *T. gondii* at a MOI of 5 or left uninfected. After a following 48 hours, samples were analysed. Each treatment was performed in triplicate and data shown above presents 6 independent experiments. Data were analysed in GraphPad Prism using a Two-way ANOVA to detect significant differences. **A)** The cell lysates were analysed using an arginase assay. A significant difference was found between stimulated and unstimulated cells, which had been infected and pre-exposed to an inhibitor and compared to expected values (****P<0.0001). A significant difference has also been found between uninfected BMDM when compared to their infected counterparts (****P<0.0001). **B)** The supernatant was analysed using an IL-6 ELISA Kit. A significant difference was found between stimulated BMDM, which have been pre-exposed to a cl-CD95L inhibitor when compared to stimulated BMDM that were not inhibited (*P<0.05). Significant differences were also found between uninfected BMDM and their infected counter parts (****P<0.0001). **C)** By preforming parasite counts a significant difference was found between BMDM stimulated with 100ng/mL of cl-CD95L which had been pre-exposed to an arginase inhibitor when compared to expected values (*P<0.05). Significant differences were also found between unstimulated and stimulated BMDM, which had not been inhibited (*P<0.05).

3.9. cl-CD95L leads to enhanced phospho-kinase expression

During our previous investigations there has been differences in responses between stimulated and unstimulated BMDM regardless of infection with *T. gondii*. To determine if these differences were caused ultimately by cl-CD95L stimulation a Proteome Profiler was utilised which assesses protein expression in cell lysates. A Phospho-Kinase Protein Array was selected to identify changes to the global profile within cellular signalling pathways which may account for the phenotypic differences we identified previously. Data was analysed using imaging software and presented in a heat map format as pixel densities. Striking differences in protein expression can be seen between unstimulated and stimulated BMDM in Figure 15.

A variety of proteins found to be upregulated by cl-CD95L are linked to signalling pathways. This included the upregulation of sphingosine 1 phosphate (S1P) signalling pathways which have previously been shown in Th17 cells stimulated with cl-CD95L. Similarly, Ca^2+ signalling pathways have also been upregulated with the addition of cl-CD95L. This pathway has been shown in previous studies to be linked with endothelial transmigration due to cl-CD95L stimulation (Tauzin *et al.*, 2011).

Figure 15 also shows that stimulation with cl-CD95L leads to upregulation of the JAK/STAT pathway in macrophages. We demonstrated that both STAT5 and STAT3 were upregulated after stimulation with cl-CD95L. Figure 15 demonstrates that STAT3 has the most prominent upregulation of all the STAT proteins, after

cl-CD95L stimulation. The activation of the JAK/STAT cascade is vital for the expansion of MDSCs (Nagata *et al.*, 2019).

The Phospho-kinase microarray showed the upregulation of MAPK pathway after stimulation of macrophages with cl-CD95L. The MAPK pathways has been found to be a key regulator of MDSC function (Trikha and Carson, 2014). The mTOR pathway has been shown to promotes MDSC recruitment, regulate induction of MDSCs, and strengthen their immunosuppressive responses (Zhang *et al.*, 2017). Figure 15 shows that cl-CD95L causes upregulation of the VEGF pathway. The activation of the VEGF pathway is key for the infiltration of MDSCs (Yang, Yan and Liu, 2018).

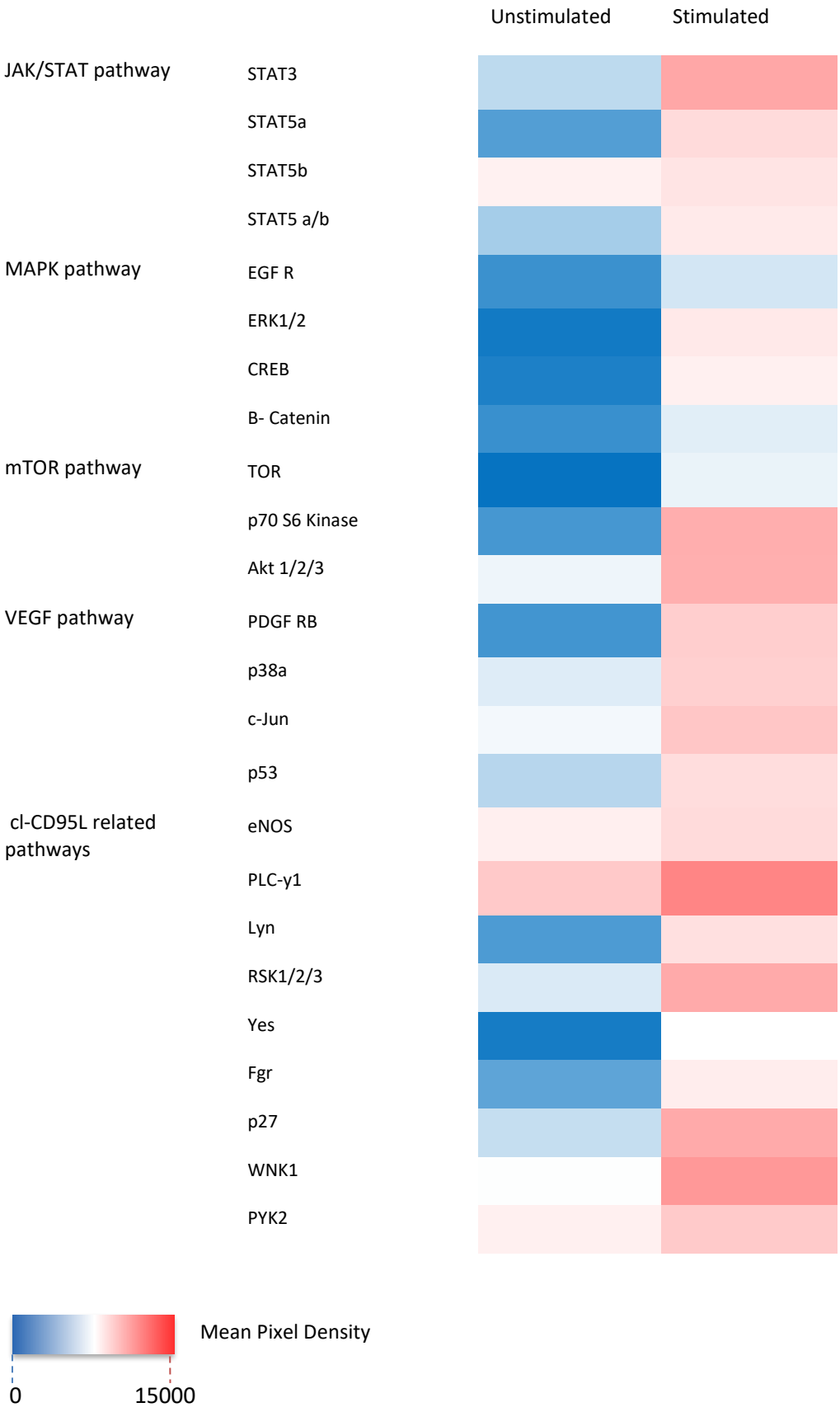

Figure 15. Heatmap illustrating the variation in protein expression by BMDM stimulated with 100ng/mL of cL-CD95L and BMDM left unstimulated

BMDM were harvested and after 24 hours either stimulated using 100ng/mL of cl-CD95L or left unstimulated. After a following 24 hours samples were analysed for protein concentration using a BCA Protein Assay Kit. Once protein concentration was determined the same concentration was used to detect the difference in protein expression in each sample using a Phospho-Kinase Array. Each treatment shown above presents 2 independent experiments. Pixel Density was analysed using ImageJ software. Data was then presented by a heat map shown was created using the averages from the two independent experiments.

Chapter 4. Discussion

Approximately 1-2 billion people are infected with *T. gondii* globally, making it one of the most successful protozoan parasites. In the United States alone 10-40% of all adults with AIDS are latently infected with *T. gondii* and one-third of those with a latent infection will go on to develop Toxoplasmic encephalitis (Luft and Remington, 1992). *T. gondii* infections cause fatal and devastating brain disease and eye disease including ocular toxoplasmosis. Ocular disease is more severe in continents like South America due to the virulent genotypes of the parasite present in this region (Carneiro *et al.*, 2013). In developing countries, the major route of infection with *T. gondii* is through untreated drinking water as opposed to in the Western world there the main infection route is through raw or uncooked meat (Petersen, Kijlstra and Stanford, 2012).

The current treatment course for *T. gondii* infection is associated with high levels of drug toxicity and often requires prolonged treatment courses ranging between weeks to over a year. The prolonged treatment time and the risk of relapsing is due to *T. gondii* tissue cysts and the lack of an efficacious drug capable of targeting this stage. There are many challenges in developing effective *T. gondii* treatments which include elimination of tissue cysts, decreasing toxicity, and practical for use in countries with poorly developed healthcare systems (Alday and Doggett, 2017).

There are currently no licensed vaccines against *T. gondii* available for human use. There is only one commercially available vaccine to protect against congenital infections in sheep. The vaccine Toxovax is based on the live attenuated S48 strain. Unfortunately, Toxovax causes adverse effects in pregnant ewes, has a short shelf-life and has a great expense. Due to Toxovax being a live attenuated vaccine it may revert to pathogenicity and is therefore not suitable for human use (Kur, Holec-Gąsior and Hiszczyńska-Sawicka, 2009). In the absence of an effective, non-toxic treatment, we believe it is critical to better understand the immunogenic ligand cl-CD95L, that may increase the pathogenicity of *T. gondii* during mammalian infections. This understanding of the immune environment created during infection could facilitate future success in host-directed therapeutics in the treatment of *T. gondii*. Host-directed therapeutics act upon the host-mediated responses to change the local environment in which the parasite exists to make it less favourable for parasite replication. To better understand the impact of cl-CD95L on *T. gondii* infections experiments were preformed using both VERO cells and BMDM.

CD95 is a ubiquitously expressed death receptor which is involved in a plethora of physiological and pathological apoptotic processes. The interaction between CD95 and its ligand induces the recruitment of signaling moclecules initiating a signalling cascade ultimately leading to cell death (Ricci-Vitiani *et al.*, 2000). Conversley, cl-CD95L does not trigger an apoptotic signalling pathway. cl-CD95L has been found to promote endothelial transmigration of T-cells aggravating clinical symptoms of experimental Lupus in mice (Poissonnier *et al.*, 2016).

To begin to investigate the role of cl-CD95L we utilised host VERO cells. The major finding of the VERO cell experiments was that 100ng/mL of cl-CD95L amplified the levels of monolayer destruction after *T. gondii* infection while also leading to increased tachyzoite numbers. Previous studies have shown that *T. gondii* processes and degrades caspase 8 which is a key initiator of apoptosis. The degradation of caspase 8 ultimately blocks CD95-mediated apoptosis which signalling is independent of the host mitochondria apoptogenic functions (Vutova *et al.*, 2007). However, *T. gondii* has also been shown to inhibit cell apoptosis by interference with the mitochondrial amplification loop (Hippe *et al.*, 2008). These studies mirror our monolayer destruction data, that when VERO cells are infected with *T. gondii* the levels of destruction are less than that in our cl-CD95L stimulated/infected groups.

From monolayer data alone, the increase in monolayer destruction in infected cells after stimulation with cl-CD95L could be due to apoptosis. However, after obtaining parasite counts, we believe that cl-CD95L leads to an increase in parasite replication, which results in the destruction of the cell monolayer. Prior studies support these findings, showing that cl-CD95L fails to elicit apoptotic signalling pathways. Similar findings have also been reported demonstrating that cl-CD95L downregulates proapoptotic activity in mice (Schneider *et al.*, 1998), with the cleaved ligand instead inducing pro-inflammatory signalling pathways (Poissonnier *et al.*, 2016).

Further support of this was evident when performing the VERO cell experiments it was also discovered that stimulation with cl-CD95L also caused increased cell viability. This effect was found when comparing non-infected cells and also when comparing cells infected with *T. gondii*. Demonstrating that cytotoxicity also decreases when VERO cells are infected and stimulated with cl-CD95L. This is not the first time cl-CD95L has been linked to cell survival and growth. Previously it has been found that stimulation with cl-CD95L leads to the upregulation of the sphingosine-1-phosphate (S1P) pathways in Th17 cells (Poissonnier *et al.*, 2016). Studies have shown that S1P has a role as a survival factor with its ability to rescue cells from apoptosis, in addition to its role in immune cell differentiation, migration and function (Reimann *et al.*, 2015). Previous research has also found cl-CD95L to be is a powerful indicator of metastatic dissemination in breast cancer (Malleter *et al.*, 2013).

Despite the important role of cell lines in research evidence has accumulated that cell lines are frequently contaminated or misidentified. In labs in Europe, the United States and Asia it has been found that at least 15% of cell lines are contaminated or misidentified. Misidentification can often go unidentified due to cells being morphologically similar. While it has been found that between 5-10% of cell culture studies have used cells which have mycoplasma contamination (Lorsch, Collins and Lippincott-Schwartz, 2014). While cell lines are a powerful tool, caution should be taken when using them in place of primary cells. Although cell lines maintain features close to primary cells it may be difficult to determine as primary cell functions are often not understood in their entireity. Furthermore,

over time serial passage of cell lines can further cause genotypic and phenotypic variation leading to heterogeneicty in cultures (Kaur and Dufour, 2012). Therefore, VERO cells may not adeuately represent results seen in primary cells. For these reasons after our preliminary studies using VERO cells, we furthered the investigation by using more immunologically significant BMDM.

Similar to the VERO cell experiments, we found that the stimulation of BMDM with cl-CD95L increases parasite burden. Therefore, we believe that cl-CD95L stimulation is detrimental to the host cell and favours parasite replication during *T. gondii* infection. To determine that the changes in BMDM responses seen was directly caused by stimulation with cl-CD95L we added a cl-CD95L inhibitor, soluble CD95-Fc chimeric protein, to our experiments. In the cl-CD95L inhibited treatments there was a decrease in tachyzoite numbers when compared to stimulated BMDMs. Similarly, during infection with *T. gondii* the levels of IL-6 and arginase levels decrease when cl-CD95L is inhibited. These inhibition experiments demonstrate that the upregulation of IL-6, arginase and parasitaemia are directly linked to BMDM stimulation with cl-CD95L.

A major finding of these experiments was the increased production of IL-6 after stimulation with cl-CD95L during infection. Previous studies have demonstrated that *T. gondii* infected patients have a twofold higher level of IL-6 when compared to healthy individuals (Matowicka-Karna, Dymicka-Piekarska and Kemona, 2009). During experiments with KO mice, studies have shown that in the absence of IL-6, mice are unable to initiate a rapid proinflammatory response to *T. gondii.* It has been suggested that increased mortaility evident in IL-6 KO mice is linked to

increased parasite burdens (Jebbari *et al.*, 1998). Contradictory to this, studies has found that pre-treatment of mouse BMDM with IL-6 leads to enhanced levels of *T. gondii* replication (Beaman, Hunter and Remington, 1994). Therfore we propose that the increase in IL-6 production after cl-CD95L stimulation may be a cause of the increased parasitemia we previously demonstrated in this study.

After demonstrating an effect of cl-CD95L stimulation on the secretion of IL-6 by BMDMs we further investigated the effects of neutralising IL-6 on BMDM. The levels of arginase activity in cell lysates of IL-6 neutralised BMDM were examined. As previously found, the levels of arginase activity increase significantly after infection. By the same assay we also demonstrated that after the neutralisation of IL-6 the levels of arginase decreased significantly in both stimulation and unstimulated BMDM when compared to the non-neutralised counterparts, the same was also true for parasite numbers. This may be due to IL-6 enhancing the expression of arginase 1 and therefore once IL-6 is inhibited the production of arginase decreases (Qualls *et al.*, 2010). This lower parasite burden has also been demonstrated in other studies in which IL-6 has been neutralised during in vivo *T. gondii* infections of C57BL/6 mice (Rochet *et al.*, 2015). From this data it can be summarised that when IL-6 is neutralised in BMDM this does not lead the expected rise in parasite counts seen after cl-CD95L stimulation. Therefore, cl-CD95L may increase parasite replication in an IL-6 dependent manner.

From our study we also demonstrated that stimulation with cl-CD95L during a *T. gondii* infection lead to an increase in arginase production in murine BMDM. Both arginase and iNOS share the same substrate, L-arginine with arginase hydrolysing L-arginine to L-ornithine and urea. The production of L-ornithine favours the growth of *T. gondii* while also being a precursor for the synthesis of L-glutamine, L-proline and polyamines. These polyamines are also essential for the proliferation of both cells and parasites. iNOS synthesises vast amounts of NO through the oxidation of L-arginine (Li *et al.*, 2012). NO is a major effector molecule in macrophage-mediated cytotoxicity and for this reason is considered to be a defence mechanism against *T. gondii* (Adams *et al.*, 1990). However, the potential parasite killing effects of high NO levels are limited by arginase competition with iNOS for the L-arginine. Interestingly, *T. gondii* is capable of infecting BMDM and reducing the production of NO (Lüder *et al.*, 2003). We speculate that *T. gondii* along with cl-CD95L increases levels of arginase while decreasing parasite limiting NO production. Furthermore, the combined effect of parasite and cl-CD95L may synergise to directly impact host cells ability to reduce parasitemia.

Macrophages can be divided into two functionally distinct subsets, classically activated macrophages (CAM) and alternatively activated macrophages (AAM) depending on the cytokine environment to which macrophages have been exposed. Exposure to IFN-γ and LPS will lead to CAMs which promote inflammation and killing of intracellular pathogens. While exposure to IL-4, IL-10, or IL-13 leads to AAMs that regulate inflammation and promote tissue repair

(Classen, Lloberas and Celada, 2009). During this study we demonstrated both an inflamatory IL-6 response commonly seen in CAMs while also showing a rise in the arginase response, a hallmark of AAMs. Therfore, we began to speculate how this seemingly paradoxical event could occur.

This induction of AAM hallmarks within a CAM macrophage would allow an efficient self-regulation of both cytotoxic and proinflammatory responses during the progression of an immune response. However, it has been shown in mice that BMDM lacking the expression of Arg1 have increased clearance of pathogens. Furthermore, the production of Arg1 in CAMs prevents competition by NO weakening BMDM ability to control *T. gondii* infections(El Kasmi *et al.*, 2008). Subsequent studies have been able to demonstrate that murine macrophages populations can respond to different stimuli and simultaneously express both CAM and AAM phenotypes in response to a *T. gondii* infection (Patil *et al.*, 2014). Thus expression of arginase within a majority CAM population would serve to limit pathology while controlling parasite load.

Another explanation maybe that cl-CD95L induces an MDSC-like phenotype in murine BMDM. It has been shown that IL-6 induces SOCS3 dysfunction and activates the JAK/STAT signalling pathway promoting the ampification of MDSCs during *in vitro* and *in vivo* studies of cancer (Jiang *et al.*, 2017). Previous studies have also demonstrated that in SLE patients the accumulation of IL-6 promotes the expression of arginase by MDSCs (Wu *et al.*, 2016) (Flynn and Legembre, 2016). The increase in arginase production by MDSCs could therfore lead to the increase in parasitemia which we have previously seen while increases in IL-6 promote an MDSC like-phenotype.

cl-CD95L has also been shown to cause a pro-inflammatory response rather than triggering an apoptotic signalling pathway, therefore these conditions may allow for unregulated parasite growth (Poissonnier *et al.*, 2016). It has also been suggested that cl-CD95L may have a role in MDSC differentiation. Moreover there is a previous mechanistic link reported between MDCSs function and Arg-1 upregulation (Gabrilovich and Nagaraj, 2009). Additionally, it has also been demonstrated that exposure to *T. gondii* peroxiredoxins elevates Arg-1 production by driving the activation of alternatively activated BMDM (Marshall *et al.*, 2011).

Before investigating impact of arginase inhibition during a *T. gondii* infection of BMDM it was first crucial to investigate the effectiveness of the inhibitor. It was discovered through performing an arginase assay that the inhibitor caused only a small decrease in the levels of arginase activity (Figure 13. A). As expected, and previously seen during this study, IL-6 levels increased after infection regardless of stimulation or inhibition. Even in light of the effectiveness of the arginase inhibitor, there was a small decrease in IL-6 secretion found in stimulated and arginase inhibited BMDM after infection when compared to their non-inhibited counterpart. During this investigation it was also found that stimulated BMDM with arginase inhibited had reduced parasite counts in comparison to the non-inhibited counterparts. This may be due the lack of arginase and therefore its ability to deplete L-arginine, which is known to inhibited IL-6 (Fan *et al.*, 2009). The decrease in parasite counts in arginase inhibited cultures also demonstrates that the minimal effect of the arginase inhibitor was biologically significant. The

cl-CD95L driven increase in parasite load leads to increased destruction of cells causing higher levels of IL-6. IL-6 has been found to initiate an inflammation amplification loop through the MDSC like phenotype in BMDM. An MDSC like phenotype also increases levels of arginase 1 leading to the increased catabolism of L-arginine. This depletion of L-arginine has been shown to promote the growth of multiple species of protozoan parasites (Iniesta, Gómez-Nieto and Corraliza, 2001). The increase in arginase production seen after BMDM stimulation with cl-CD95L could suggest the alternate activation of the macrophages. However, AAM would also cause an anti-inflammatory response not evident by the rise in IL-6. In our study we consistently demonstrated a proinflammatory response in BMDM due to stimulation with cl-CD95L and *T. gondii* infection, as well as increased levels of arginase production after stimulation with cl-CD95L. Taking this proinflammatory response together with upregulated arginase production into consideration we speculate that stimulation with cl-CD95L leads to BMDM expressing an MDSC-like phenotype or the coexistence of both CAM and AAM.

Upstream of Arg1 lies the transcription factor STAT6, studies have shown that the induction of the Arg-1 gene by IL-4 requires the direct interaction of the Arg-1 promoter with STAT6 (Gray *et al.*, 2005). To better understand the role of STAT6 in the functioning changes caused by cl-CD95L we used an inhibitor in our studies. The major finding of these STAT6 inhibited experiments was the levels of both IL-6 and Arg1 dropped to undetectable levels even after infection with *T. gondii* and stimulation with cl-CD95L. Similarly, parasite counts decreased dramatically in inhibited/stimulated cultures when compared to non-inhibited/stimulated. The data demonstrates that the inhibition of STAT6 also eliminates the upregulation previously seen in IL-6 and Arg1 after stimulation with cl-CD95L. It has been well documented that *T. gondii* inject ROP16 kinase into host cells where it activates STAT6 (Butcher *et al.*, 2011) avoiding host immunity. It has also been shown that STAT6 KO mice are less susceptable and exhibits lower parasite numbers when orally challanged with *T. gondii* compared to wild type mice (Jensen *et al.*, 2013). Therefore we also support previous findings that STAT6 hinders host abilities for parasite clearance.

To gain a deeper understanding of the impact of cl-CD95L on signalling in BMDM without infection with *T. gondii* we performed a protein signalling array.

A major finding in the investigation is the upregulation of signalling pathways integral to MDSCs, this includes the increased expression in the JAK/STAT pathway. Studies have shown most signalling pathways that are triggered by MDSCs converge on STAT3 and JAK protein family members (Pyzer *et al.*, 2016). The upregulation of STAT3 and STAT5 allows regulation of MDSC expansion, during our study the stimulation of BMDM with cl-CD95L leads to increased STAT expression, as demonstrated by the effects of STAT6 inhibition.

The Akt signalling pathway was upregulated through BMDM stimulation with cl-CD95L. Akt exists as three isoforms in mammals, Akt1 is widely distributed through tissues, while Akt2 is found in fat and muscle cells, and Akt3 is present prefominantly in the brain. The Akt pathways regulates a multitude of biological processes, these incude cell growth and proliferation (Hers, Vincent and Tavaré, 2011). ATK can lead to the activation of mTOR, mTOR belonging to a group of Ser/Thr protein kinases of the PI3K superfamily (Liu *et al.*, 2009). We also demonstrated that stimulation of BMDM with cl-CD95L leads to the upregulation of mTOR. mTOR plays a pivotal role in the regulation of cell growth and proliferation by monitoring nutrient availability, cellular energy levels, oxygen levels, and mitogenic signals. mTOR has previous links with cancer, as activation of mTOR provides a growth advantage to tumour cells by promoting protein synthesis (Liu *et al.*, 2009). The Ras-ERK and PI3K-mTOR pathways are also critical in controlling cell survival, differentiation and motility. ERK which is a MAPK functions as a major effector of the Ras oncoprotein. These pathways can

negatively affect each other's activity or activate signalling (Mendoza, Er and Blenis, 2011).

During BMDM stimulation with cl-CD95L we found an increased expression of members of the VEGF signalling pathway. Previous research has demonstrated that VEGF can decrease apoptosis caused by CD95 interacting with its ligand (Berkkanoglu *et al.*, 2004). VEGF expression has also been shown to promote tumour angiogenesis and immunosuppression in ovarian cancer. This is consistent in studies analysing triple-negative breast cancer where an inverse correlation between VEGF and T-cell function has been found. A correlation has also been found between VEGF expression and MDSC infiltration (Horikawa *et al.*, 2017). Again highlighting the potential link between cl-CD95L and MDSC induction.

Importantly, the data we obtained from our array mirrored studies that identified signalling pathways involving cl-CD95L previously. Previous studies have shown that cl-CD95L up regulates c-yes and PI3K-driven signalling pathways, these pathways are used by cl-CD95L to promote cell migration (Tauzin *et al.*, 2011). A mechanistic link has previously been demonstrated in triple-negative cancers between c-Yes and PI3K pathways (Malleter *et al.*, 2013). In these studies cl-CD95L levels are elevated and cl-CD95L has therefore been demonstrated to be is a potent marker for metastatic dissemination (Legembre *et al.*, 2014). Another key cl-CD95L pathway which we also found to be upregulated in our study is the calcium signalling pathway through expression of PLC-y1. Previous studies have shown that both the activation of the PI3K pathways and the rise of intracellular calcium play a major role in achieving cell motility (Tauzin *et al.*, 2011).

This understanding of the effect cl-CD95L has during *T. gondii* infections is critical due to links between the parasite and the activation of matrix metalloproteases (Seipel *et al.*, 2010). CD95L is cleaved by MMPs during infection with *T. gondii* leading to more cl-CD95L circulating in the host amplifying an inflammatory loop. Figure 16 illustrates the pathways found to be upregulated during our study when macrophages are infected with *T. gondii* and stimulated with cl-CD95L. It was demonstrated that a proinflammatory response was still present in macrophages while there is also an upregulation of Arg-1 in a STAT6 dependent manor. This study shows that when IL-6, Arg-1 and STAT6 are upregulated the conditions are favourable for parasite replication.

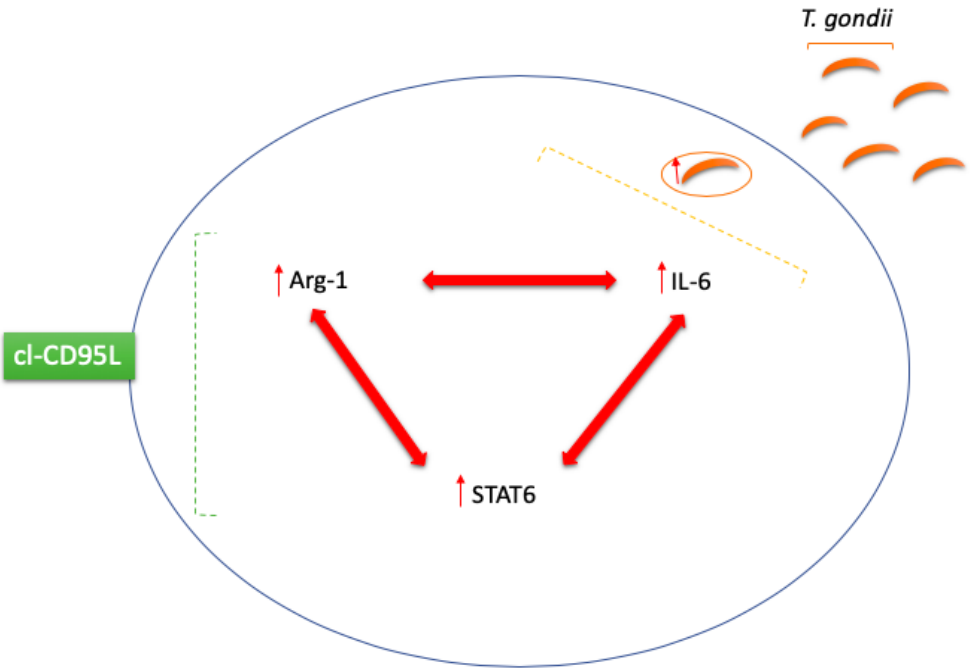

Figure 16. Diagram showing interactions during macrophage infection with *T. gondii* and cl-CD95L.

To refine our hypothesis that cl-CD95L is causing non-apoptotic signalling, a more sensitive assay could be used such as PI staining. Using PI staining measurement by flow cytometry it would also be possible to associate cell viability with parasite invasion if we were also capable of using a fluorescent strain of *T. gondii.* Alternatively, monitoring caspase activity which sits downstream of CD95 apoptotic signalling would also verify that known CD95 signals were being altered.

Expanding the pool of samples analysed by the Phospho-kinase array to demonstrate the pathways upregulated during a *T. gondii* infection as well as stimulation with cl-CD95L. Work leading on from this would include an element of validating individual targets which are implicating in MDSC biology including IFN-γ, TGF- β, and IL-17. This would complement studies, to allow for a more robust identification of MDSCs, flow cytometry should be used to phenotype the cells population.

Combining those studies could lead to an *In vivo* study to measure levels of cl-CD95L circulating in mice during a *T. gondii* infection, levels of cl-CD95L can be analysed using an ELISA. Following on from this it would also be interesting to inhibit cl-CD95L with mFas-Fc Chimera *in vivo* to investigate if this creates a less favourable environment for parasite replication.

Chapter 5. Concluding remarks

The overarching aim of this study was to identify what, if any, mechanisms by which cl-CD95L impacted host cell survival during a *T. gondii* infection. Previous studies have demonstrated that cl-CD95L prevents apoptosis and promotes cell viability and in both cancer and lupus, cl-CD95L has detrimental effects on host immune responses.

The aim of our study was to determine if cl-CD95L also has an adverse effect on host immunity during a *T. gondii* infection. We demonstrated that cl-CD95L stimulation leads to a greater pro-inflammatory response then that caused by *T. gondii* alone. We also determined that cl-CD95L stimulation upregulates Arg-1 expression in a STAT6 dependent manner. Taking these individual observations, we speculate that cl-CD95L stimulation primes BMDM to behave like MDCS. In our study we have shown that the stimulation of BMDM with cl-CD95L leads to an MDSC-like phenotype. Figure 16 illustrates the similarities in traditional MDSC expression and the findings of our study.

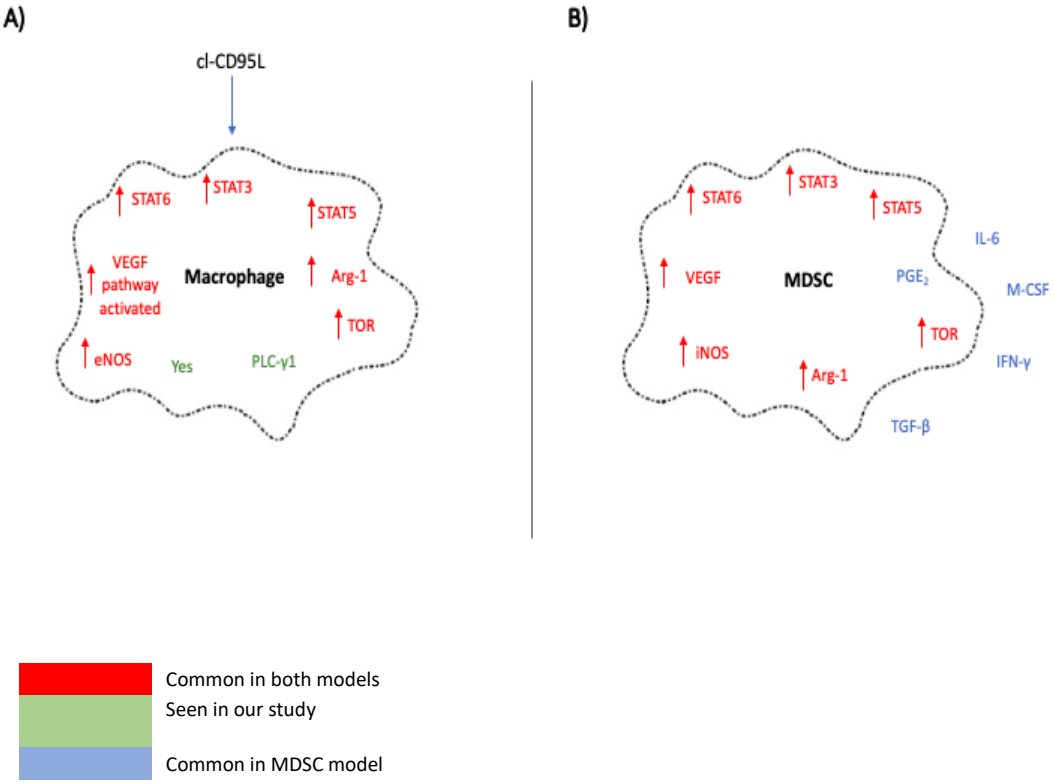

Figure 17. Illustrating similarities between cl-CD95L treated BMDM and MDSCs

A) Depicts the BMDM response following cl-CD95Lstimulation incorporating both phosopho-kinase array results from infected/stimulated experiments
B) A general MDSC response is shown with both effector and promoter cytokines alongside intracellular signalling factors which have been previously been associated with the presence of these cells.

Chapter 6. References

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Appendix 1

1.1. Recipes

1.1.1 ELISA

Wash Buffer

- KCl- 0.2g
- NaCl- 8g
- Na_2HPO_4- 1.44g
- KH_2PO_4- 0.24g
- DI water- 800mLs
- Tween-20- 500µLs

Bicarbonate/Carbonate Coating Buffer

- Na_2CO_3- 3.03g
- $NaHCO_3$- 6.0g
- DI water – 1,000mLs

Diluent

- NaCl- 8.0 g
- Na HPO- 1.16 g
- KH PO- 0.2 g
- KCl- 0.2 g
- Add deionized water to 1.0 L

Acid Stop Solution

- H_2SO_4 -0.16M

1.1.2. Arginase assay

Arginase Activator (50mM)

- Tris-HCl- 7.88g
- DI water- 1,000mLs
- Set pH to 7.5

10mM $MnCl_2$/ 50mMTris-HCl

- $MnCl_2$- 0.126g
- Tris-HCl- 100mLs

L-arginine Substrate

- L-arginine- 0.871g
- DI water- 10mLs

Acid-stop Solution

- H_2SO_4- 1mL
- H_3PO_4- 3mLs
- DI water- 7mLs

Alpha-ISPF

- α- ISPF- 0.09g
- 100% ethanol- 1mL