Abstract

Cryptosporidiosis is a common infectious diarrhoeal disease of mammalian livestock and humans worldwide. The etiological organisms responsible are intestinal apicomplexans of the genus *Cryptosporidium*, including *C. parvum*, that infect intestinal epithelial cells. Immunocompromised or malnourished hosts develop severe life-threatening disease. Immunological elimination of *Cryptosporidium* requires $CD4^+$ T cells and IFN-γ. Nevertheless, studies have shown innate immune responses have a significant protective role. Importantly, in T cell-deficient mice, IFN-γ is important for control of *C. parvum* infection. In innate immunity natural killer (NK) cells are major producers of IFN-γ and are activated by cytokines including type I IFNs but the roles of these components in immunity to *Cryptosporidium* infection have not been investigated. Therefore, the purpose of this project was to study the involvement of type I IFNs and NK cells in immunity to *C. parvum* employing *in vitro* and *in vivo* (murine) infection models.

Enterocytes were shown capable of the production of type I IFNs in response to *C. parvum* infection. These cytokines directly inhibited parasite development in epithelial cells. Also, in neonatal SCID mice the level of infection increased after treatment with anti-type I IFN neutralising serum. A higher level of infection was observed in $Rag2^{-/-}\gamma c^{-/-}$ mice deficient in T, B and NK cells in comparison to $Rag2^{-/-}$ mice with a normal NK cell population and early mortality during chronic infection of adult animals was associated with the absence of NK cells. Using cultures of SCID mouse splenocytes, NK cells were the main source of IFN-γ in response to *C. parvum* antigen stimulation. However, IFN-γ was also found to have a protective role in $Rag2^{-/-}\gamma c^{-/-}$ mice, implying cells other than lymphocytes produce this cytokine.

In conclusion, this is the first study to indicate important protective roles for type I IFNs and NK cells in innate immunity against *C. parvum.*

List of Contents

List of Tables

List of Figures

Acknowledgment

Completing one journey, my MSc, I embarked on another. The MD(Res) has proved to be an experience like no other, more challenging but also incredibly rewarding.

Once again I am indebted to many people for helping me reach this point in my journey and the list of acknowledgments is long.

I must begin by thanking my 'Boss', Dr Vincent McDonald. Firstly, for inviting me to join his group and continuing to be my supervisor (after the MSc). The diverse lab techniques and animal experimentation that he taught me have been invaluable. He constantly stimulated scientific thinking and argument. He also guided me through the writing process. As well as being my supervisor, he has been a true friend; assisting and supporting me whenever I needed it. My life would have been very different without him and his trust in me.

I am grateful to Professor Ian Sanderson, for allowing me to join the Gastroenterology team, and his supervision and constant support in the scientific and clinical fields.

Professor Graham Foster, an International clinical expert of Interferon therapy in hepatitis, for his kind help and guidance with the interferon work.

Dr Nick Croft, 'My clinical supervisor', for encouraging and facilitating all the clinical aspects of my MD(Res) training. I am privileged to have been trained by him and would have never been the clinician I am now without his support. I am really grateful to him for everything.

Dr Anne Ballinger, for her financial help without which this project could not have started before BBSRC funding eventually arrived.

To my parents, there are no words to describe my gratitude. You have always accepted me for whom I am; believed in me and gave me everything you had, and more. Without you, I would not have been able to do any of this. I know I will never be able to repay you but I will try my hardest not to let you down. You are the greatest parents and I love you so much.

Ammar, my dear brother; despite his initial reservations about my decision to take this long path, he supported and stood by me, I can't thank him enough. Wish him luck with his own research work.

Asil, I continue to thank her for helping me to come to London and join the research world; but more importantly for surrounding me with the love and the special care that a big sister would give.

Habib, my best friend, like this degree journey our friendship has been through many happy and difficult times. The obstacles have made me learn more about myself and pushed me further to achieve my goals. Thank you for encouraging me to do better and for teaching me to smile in the face of adversity.

Post Docs and PhD students; especially Daniel Korbel for his exceptional efforts and help. Dr Bo Zo for his help with the FACS and MACS techniques. Also, Paul Creeke Naheed, Jenny, Alistair, and Meera. Sangeetha 'my research mate' for being there and

sharing many moments, both good and bad. I wish all of them the very best in the future.

Dr Ping Wang for valuable advises and support.

Ms Jacqui Frith for her endless support. Her happy energy and efficiency have always made the voyage easier. Nici and Anna, for making me feel cared for.

My best friends Nada and Tagi, for always being there for me. Thanks for keeping me inspired, and congratulations on their own achievements.

Rehab (MSc student), meeting her was one of the unexpected gifts that this expedition offered. I am lucky to have known her.

Holly, for helping me with the final formatting of this thesis. Hope to see her studying medicine soon.

To all the friends and family members not mentioned here, who supported me, thank you.

To everyone, my utmost gratitude for helping me through this journey.

Finally, I dedicate this thesis to immunocompromised and malnourished children everywhere. My great wish is that the outcomes of our research efforts lead to therapies that will ameliorate their suffering.

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CHAPTER ONE

GENERAL INTRODUCTION

SECTION I: REVIEW OF *CRYPTOSPORIDIUM* AND CRYPTOSPORIDIOSIS

1.1.1 Introduction

Cryptosporidium is a protozoan parasite that was first discovered by Tyzzer in 1907 in the gastric glands of mice (Tyzzer, 1907) but it was not until 1976 that the parasite was described as a pathogen in humans and by the end of the 20th century it became accepted as a significant zoonotic pathogen. It has been considered a great public health problem after a large human waterborne outbreak in Milwaukee in 1993 when over 400,000 residents were affected (Mackenzie *et al*, 1995).

The importance of *Cryptosporidium* is increasingly being recognized and it was identified as a neglected pathogen in the World Health Organization's Neglected Diseases Initiative 2004.

Cryptosporidiosis is self-limiting in immunocompetent patients but severe and life threatening in immunocompromised individuals. In developing countries, cryptosporidiosis remains a serious risk as a frequent cause of malnutrition and death in young children. Treatment options are limited and, therefore, prevention and control measures are important for the protection of vulnerable groups.

1.1.2 Taxonomy

The genus *Cryptosporidium* belongs to the Superkingdom Eukaryota, Phylum Apicomplexa, Class Sporozoasida, Subclass Coccidiasina, Order Eucoccidiida, Suborder Eimeriina, Family Cryptosporiidae (Plutzer and Panagiotis, 2009).

However, some studies have suggested that the genus *Cryptosporidium* belongs to an early emerging lineage of the Apicomplexa that is closer to Gregarinia than to the Coccidia, based on a number of characteristics, including the oocyst auto-infectivity, the nature of the association with the host cells and the parasite insensitivity to anticoccidial drugs (Barta and Thompson 2006, Hijjawi et al 2004). Table (1.1) summarise the differences between *Cryptosporidium* and Coccidia. Further studies are required to confirm the taxonomy of the parasite.

Table 1. Differences between *Cryptosporidium* and the Coccidia (Barta and Thompson, 2006)

Property	***Cryptosporidium***	**Coccidia**
Location within the cells	**Intracellular but extracytoplasmic**	**Intracellular**
Attachment or feeder organelle	**Present**	**Not present**
Morpho-functional types of oocysts	**Two types: Thick and thin-shelled**	**Thick shelled**

Size of oocysts	**Small (5-7.4×4.5-5.6μm)**	**Larger (9-38×7-39 μm)**
Sporocyst, micropyle and polar granules in oocysts	**Lacking**	**Present**
Extracellular development	**Yes (This is highly controversial)**	**No**
Syzygy-like pairing of extracellular gametes	**Yes (This is highly controversial)**	**No**
Apicoplast	**Absent/Lost**	**Present**
Complexity of biosynthetic pathways	**Simplified; reliant on salvaging from host**	**More complex**
Sensitivity to anticoccidial drugs	**Insensitive**	**Sensitive**
Host specificity	**Low for some species**	**High**
Pathogenesis	**Not understood**	**Mainly understood**

Twenty species of *Cryptosporidium* are now accepted based mainly on genotypes, phenotypes and host varieties. These are shown in Table 2 (Plutzer and Karanis, 2009) and at least eight (*C .hominis, C. parvum, C. meleagridis, C. felis, C*. *cani*s, *C. suis, C. muris* have and*C. andersoni*) have been detected in humans.

Table 2.*Cryptosporidium* species (Fayer 2008; Plutzer and Panagiotis 2009)

Species	**Author**	**Main Host**
C. andersoni	**Lindsay *et al*. (2000)**	***Bos taurus* (domestic cattle)**
C. baileyi	**Current *et al*. (1986)**	***Gallus gallus* (chicken)**
C. bovis	**Fayer *et al*. (2005)**	***Bos taurus* (domestic cattle)**
C. canis	**Fayer *et al.* (2001)**	***Canis familiaris*** **(domestic dog)**
C. fayeri	**Ryan *et al*. (2008)**	***Macropus rufus*** **(red kangaroo)**
C. felis	**Iseki (1979)**	***Felis catis* (domestic cat)**

C. fragile	**Jirku *et al.* (2008)**	***Duttaphrynus melanostictus*** **(black-spined toads)**
C. galli	**Pavlasek (1999)**	***Gallus gallus*** **(chicken)**
C. hominis	**Morgan-Ryan *et al.* (2002)**	***Homo sapiens*** **(human)**
C. macropodum	**Power and Ryan (2008)**	***Macropus giganteus*** **(grey kangaroo)**
C. meleagridis	**Slavin (1955)**	***Meleagris gallopavo*** **(turkey)**
C. molnari	**Alvarez-Pellitero and Sitja-Bobadilla (2002)**	***Sparus aurata*** **(gilthead sea bream)**
C.muris	**Tyzzer (1910)**	***Mus musculus*** **(house mouse)**
C. parvum	**Tyzzer (1912)** **Nime et al. (1976)**	***Mus musculus*** **(house mouse)** ***Homo sapiens*** **(human)**
C. ryanae	**Fayer et al.(2008)**	***Bos taurus*** **(domestic cattle)**

C. scophthalmi	**Alvarez-Pellitero *et al* (2004)**	***Scophthalmi maximus* (turbot)**
C. serpentis	**Levine (1980)** **Brownstein *et al.*(1977)**	***Elaphe guttata* (corn snake)** ***Elaphe subocularis* (rat snake)** ***Sanzinia madagascarensus* (Madagascar boa)**
C.suis	**Ryan et al. (2004)**	***Sus scrofa* (domestic pig)**
C. varanii	**Koudela and Modry (1998)**	***Varanus prasinus*** **(Emerald monitor)**
C. wrairi	**Vetterling *et al*. (1971)**	***Cavia porcellus* (guinea pig)**

1.1.3 Epidemiology

Cryptosporidium has been reported to infect people in 106 countries both in immunocompetent and immunocompromised individuals and in developed and developing countries (Fayer, 2008). Many outbreaks of infectious diarrhoeal disease have been reported to be caused by this genus (Casemore *et al.,*1997)

In the UK *Cryptosporidium* is the commonest protozoal cause of gastroenteritis, with 3000-6000 annually confirmed laboratory cases. *C. parvum* and *C. hominis* represent most of laboratory confirmed cases (Davies and Chalmers, 2009). Cryptosporidiosis inEngland and Wales are seasonal with peaks both in spring and autumn and while *C. parvum* is more common in spring, *C. hominis* is more prevalent in late summer and autumn. The risk factors for infection are not clearly identified (Davies and Chalmers, 2009).

In the United States, during 1995-2007 the total number of reported cases of cryptosporidiosis increased from 2972 to 11,657 with a dramatic increase (4/100,000 population) from 2005 that has continued through 2007; 41.5% of reported cases were outbreak related (Yoder and Beach, 2009). Again the reason for this change is unclear.

1.1.4 Epidemiological and transmission characteristics

The *Cryptosporidium* parasite is distinguished with special and remarkable features that are critical to the spread and epidemiology of the disease and Table 3 summarises these characteristics (From Dillingham *et al.,* 2002)

Table 3. Epidemiological and transmission characteristics of *Cryptosporidium*(Dillingham *et al.*, 2002)

Characteristic	Epidemiological significance
1. Chlorine, iodine and related acid resistance.	Readily spread in chlorinated water or swimming pools and acidic foods
2. Relatively small size as it is one-third the size of *Giardia* or amoebic cysts.	Difficult to filter; hazard to the water treatment industry
3. Low infectious dose (infection can happen with as low as 10-30 oocysts in healthy individuals).	Easily acquired with high infection rates (e.g. Milwaukee drinking water supply, day care centres, hospitals, households)
4. Fully infectious when shed	Easily spread person-person (e.g. households, hospitals, day-care centres)

Under favourable conditions including (high humidity, temperatures <20°C) the oocysts can survive in the environment for about 6 months but after that infectivity rapidly decreases (Fayer *et al*., 1998). Neither moderate freezing nor heating to 50°C completely inactivates oocysts (Olson *et al.,* 1999).

1.1.5 Means of transmission

a. Drinking water

The largest outbreaks of cryptosporidiosis are associated with contamination of (fully chlorinated) drinking water by sewage effluent or manure. Private water supplies located in farms or rural areas were the cause of six outbreaks in England and Wales (Said *et al.*, 2003)

b. Recreational water

Many outbreaks have been linked to swimming in lakes and swimming pools, and often related to faecal accidents, defective filtration and sewage contamination of pool water as well as the parasite's high resistance to chlorination (Fayer *et al*. 2000).

c. Person-to-Person

Transmission is common within families, also in nurseries, day care settings, hospitals and schools (Teresa *et al.,* 2006). Sexual transmission has been also suggested (Pederson *et al.,* 1996) and a study in homosexual men in Australia identified sexual behaviour as a risk factor for cryptosporidiosis (Hellard *et al.,* 2003).

d. Zoonotic (animal to person)

This has been confirmed by many studies involving pets, farm animals and by accidental infection of veterinary workers (Current *et al.,* 1983). In addition there have been links between bovine genotype 2 cryptosporidiosis in human exposure to cattle in farms. Human infection with cat, dog and turkey genotypes also implicated zoonotic transmission (Newman *et al*., 1994).

e. Food

Food-borne infection is less common but can be caused by contaminated fruit or vegetables or food washed in contaminated water (Millard *et al*., 1995) Unpasteurised milk may be another means of parasite transmission (Gelletlie *et al*., 1997).

1.1.6 Life cycle

Cryptosporidium is monoxenous and the parasite is transmitted from host to host via the faecal-oral route (Fig 1). Infected hosts shed mature oocysts with faeces to contaminate the environment, water or food. The oocysts are tiny spore-like bodies consisting of four comma- shaped sporozoites surrounded by a tough wall that can maintain their infectivity under cool moist conditions for months. After the oocysts are ingested by other suitable hosts excystation occurs as a result of triggers provided by conditions in the intestine to release motile sporozoites that can invade the epithelial cells of the gastrointestinal tract. The sporozoites and subsequent developmental stages are located at the luminal surface of epithelial cells, establishing an intracellular but extracytoplasmic position within a parasitophorous vacuole (PV). An attachment or feeder organelle develops at the interface of the apical pole of the sporozoite and the host cell cytoplasm and the sporozoites rapidly differentiate into spherical trophozoites. An asexual form of multiplication then occurs, forming two types of meronts. Type I contains 6-8 nuclei, and when it becomes mature merozoites are released and in new host cells develop either into a type I meront again, or into a type II meront which contains 4 merozoites when mature.

The merozoites from type II meronts also invade new host cells but initiate sexual development, differentiating into either male (microgametocyte) or female (macrogamont) stages. At maturation, the microgametocytes contain sperm-like microgametes which fertilize the macrogamonts when they find an infected cell. The

fertilized macrogamont, or zygote, then develops into an oocyst which undergoes meiosis within the host cell.

When sporogony (meiotic sporozoite formation) is complete each oocyst contains four potentially infective sporozoites. Some oocysts with thick walls are shed via the faeces, while others which have a thin wall release sporozoites within the host and these may repeat the developmental cycle ie autoinfection (O'Donoghue, 1995).

Like the typical coccidians, *Cryptosporidium* by being intracellular is protected during development from elements of the host immune response and the environment of the gut, while receiving energy and nutrition from the host cells. Also, like other coccidians, it lies within a PV bounded by a parasitophorous vacuolar membrane (PVM), which in other coccidians, is the route through which nutrients enter the parasite. In *Cryptosporidium*, thePVM may play a protective role and may be selectively permeable to certain molecules from the gut lumen. The unique feeder organelle at the interface with the cell cytoplasm is often stated to be the site for nutrient uptake from the host cell, although there is no strong evidence for this (Fayer, 2008).

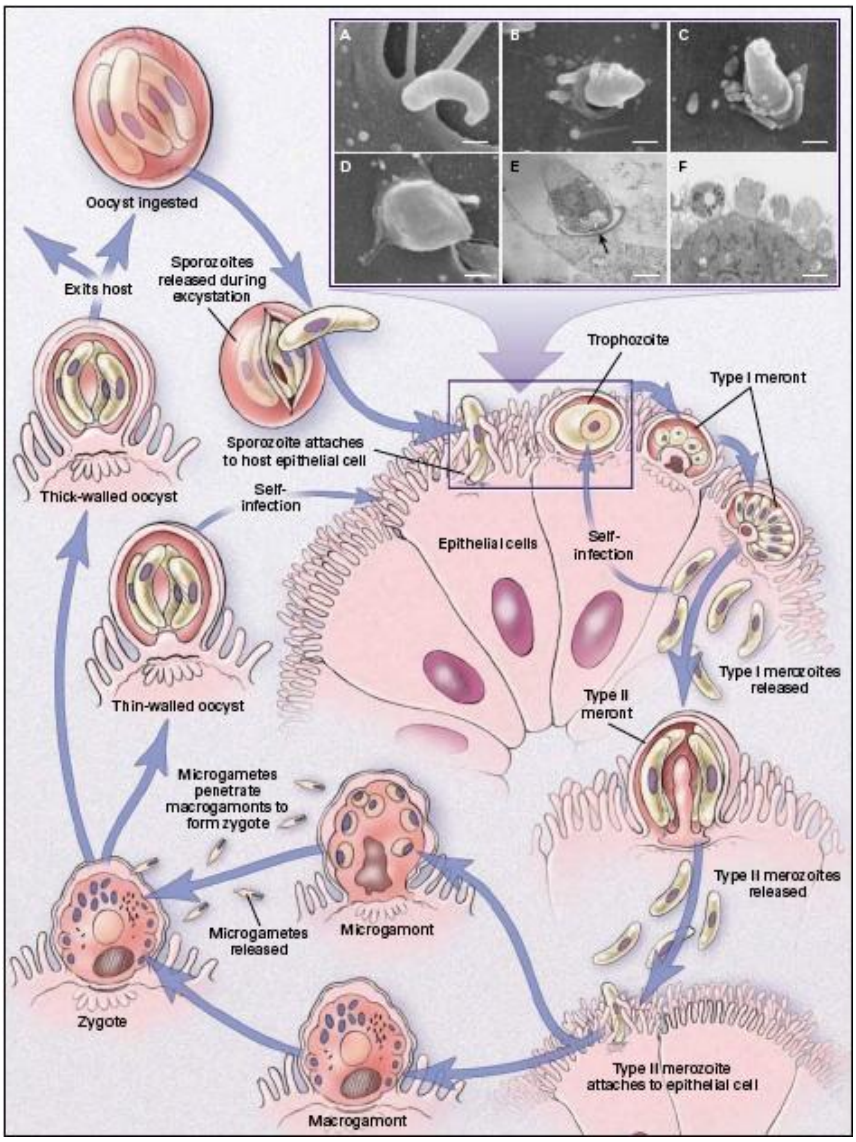

Figure 1 Life cycle of *Cryptosporidium* and infection of host cells

Chen *et al,* New England Journal of Medicine (2002).

After the oocysts are ingested, they release sporozoites, which then invade epithelial cells in the gastrointestinal tract. After entry into the epithelial cells, the sporozoites mature into trophozoites, which then reproduce in two cycles. In the asexual cycle the organism undergoes asexual reproduction (schizony), producing merozoites (type I), which are emitted into the lumen of the intestine and infect other gastrointestinal epithelial cells. In the sexual cycle, some of the merozoites (type II) attach to epithelial cells, mature into gametocytes, which are fertilized in the intestinal tract, and then form into oocysts. The oocysts then release sporozoites that can either reinfect the intestinal epithelium and start a new life cycle or be shed in feces, capable of infecting others.

Panels A through to E show a *C. parvum* sporozoite attaching to and involving a host epithelial cell *in vitro.* Panel A: sporozoite attaching the apical membrane surface of the cell. Panels B and C: sporozoite invading a host cell and the protrusion of the epithelial cell membrane around it at its attachment site. D and E: an organism enveloped by the host cell membrane and the formation of vacuole. In panel E, the zoite has made contact with the microvillous border of the epithelial cell, with its anterior end inserted in the host cell membrane (arrowed). A dense band is formed where the parasite meets the cell. Panel F shows an intestinal biopsy specimen from a patient with intestinal cryptosporidiosis.

1.1.7 Human cryptosporidiosis

1.1.7.1 Pathogenesis

The main site of infection is the small intestine, although infection may be present throughout the gastrointestinal tract and in immunodeficient hosts may spread to extra-intestinal sites. Clayto *et al.* found that in HIV patients proximal intestine involvement was associated with severe diarrhoea compared to intermittent diarrhoea or asymptomatic infection in the case of colonic infection with no small intestine involvement (Clayto *et al.,* 1994).

Invasion of the host cells is restricted to the luminal border of the enterocytes and leads to displacement of the microvilli border and loss of the surface epithelium, causing changes in the villous architecture and lymphocyte infiltration in the lamina propria (Farthing, 2000).

Osmotic, inflammatory and secretory aspects of diarrhoea have all been investigated and many factors were found to contribute to the pathogenesis (Farthing, 2000). The secretory-like diarrhoea seen in cryptosporidiosis is suggestive of enterotoxin(s) activity as a specific mechanism. This was indicated by electrolyte analysis of stools from infected children (Guarino *et al.,* 1997), but not in case controlled perfusion studies (Kelly *et al.,* 1996). Despite indirect evidence for toxin-like activity *invitro,* no parasite toxin has yet been isolated (Gaurino *et al.,* 1994, 1995).

Osmotic dysregulation which is believed to be the primary cause of diarrhoea characterised by enterocyte malfunction has been explained in an experimental infection model by the decreased absorption of glucose-coupled Na^+ caused by villous blunting and increased Cl^- secretion due to crypt hyperplasia (Argenzio *et al*., 1990). A possible mechanism of pathogenesis is that the infection with *Cryptosporidium* damages the enterocytes and eventually leads to their death that then would trigger epithelial cell hyperplasia. Apoptosis of uninfected cells of

intestinal cell lines by the parasite was confirmed by *in vitro* study(Griffiths *et al.,* 1994), but this has not been observed in infected piglets (Foster *et al.,* 2012). Changes to the microvillous border also lead to the loss of membrane-bound digestive enzymes, reduction in the absorptive surface and uptake of fluids, electrolytes and nutrients (Giffiths *et al.,* 1994).

Increased inflammation in the lamina propria could partly explain the secretory diarrhoea by the production of cytokines and neuropeptides (Laurent *et al.,*1997; McDonald *et al.,*2000). TNF-α or other cytokines may stimulate fibroblasts and other cells to secrete prostaglandins (e.g. PGE2) and these products will then enhance secretion and impair absorption (Griffiths, 1998). Faecal leukocytes are always absent although in studies of malnourished children, cytokines and lactoferrin were found in stool and infection has been associated with persistent systemic inflammatory response. (Kirkpatrick *et al.,* 2006).

1.1.7.2 Clinical presentation

Cryptosporidium can cause a spectrum of disease from asymptomatic to mild to severe, following an incubation period of 3-14 days that depends on both host (age, presence of maternal antibody, previous exposure or the infectious dose) and parasite (origin and age of oocysts, species and genotype) (Tzipori and Ward, 2002).

1.1.7.2.1 Asymptomatic infection

Hunter *et al.* (2004) compared symptomatic and asymptomatic infections in 13 studies in Denmark, Finland, Norway and Sweden. The prevalence of infection in asymptomatic people was 0.99% compared to 2.91% in patients with symptoms. In developing countries with poor sanitation, asymptomatic carriage can be higher, as shown in a study in Bolivia in which 31.6% of people carried

Cryptosporidium(Esteban *et al.,* 1999). Asymptomatic may be free of symptoms but, especially in endemic areas, asymptomatic infection might be associated with other sequelae such as failure to thrive in children.

1.1.7.2.2 Symptomatic infection

Cryptosporidiosis presents as a gastroenteritis and the differential diagnosis usually involves other causes of infectious gastroenteritis such as *Giardia*, *Cyclospora*, *Isospora* (or *Cycloisospora*), norovirus, rotavirus, *Campylobacter, Salmonella, Shigella* and *enterohaemorrhagic Escherichia coli* such as *E.coli* O157. Symptoms start with sudden onset of watery voluminous diarrhea with 3-6 stools per day (but sometimes many more) that can be offensive in smell and may contain mucous (Casemore, 2000). Pus, blood and faecal leucocytes are not typically present, however Hunter *et al.* (2004) have reported 11% of cases had bloody diarrhoea, but this is probably due to co-infections which were not identified in that study. Other symptoms that accompany diarrhoea are: abdominal cramps (in 96% of patients), nausea or vomiting (65%), mild fever (59%), anorexia, malaise and fatigue and weight loss (Hunter *et al.,* 2004). In some cases bloating and gas production can be reported (Chalmers and Davies, 2009).

In developing countries cryptosporidiosis is associated with significant morbidity and infant mortality (Molbak *et al.*, 1993) and with malnutrition in children (Sarabia-Arce *et al*., 1990). However it is difficult to establish whether the malnutrition was the causative factor in cryptosporidiosis or vice versa (Chalmers and Davies, 2009).

1.1.7.3 Long term effects

Little is known about the long term effect of cryptosporidiosis. A case control study has shown that infection with *C. hominis* (but not *C. parvum*) was associated with joint pain, eye pain, headache and fatigue two months post-infection (Hunter *et al*., 2004). Seronegative reactive arthritis has been reported in adults and children (Hay *et al* 1999; Shepherds *et al.,* 1989). One report of Reiter's syndrome (arthritis,

conjunctivitis and urethritis) has been reported (Corn and Sherry, 1995). A study has also suggested that cryptosporidiosis infection may cause relapse of inflammatory bowel disease (Manthey *et a*l., 1997).

1.1.7.4 Cryptosporidiosis in immunocompromised patients

While cryptosporidiosis in healthy, well nourished, immunocompetent patients is usually self-limiting, immunocompromised individuals can suffer prolonged severe, chronic and life threatening symptoms. Patients who are at most risk are those with T cell immune deficiency, including patients with haematological malignancies (mainly children), HIV patients with low CD4 cell counts (particularly less than 50), patients with primary T cell deficiencies such as SCID and CD40 ligand deficiency (hyper IgM syndrome) (Hunter and Nicholas, 2002). Cryptosporidiosis for those groups of patients may in addition to the typical presentation present atypical and extra-intestinal disease. The infection can affect the entire gastrointestinal tract including the pancreas and gall bladder. Complications of pancreato-biliary infection can be pancreatitis, sclerosing cholangitis and, rarely, subsequent biliary cirrhosis (Rosario de Souza *et al.,* 2004; Davies and Chalmers, 2009).

Infection of the biliary tree acts as a reservoir from which intestinal cryptosporidiosis may relapse and it has been suggested that the billiary tree may not be reached by non-absorbable anti-parasitic agents which give the organisms their resistance to the anti-parasitic agents (Baishanbo *et al*., 2006)

Tracheo-bronchial involvement and sinusitis have also been described (Dunand *et al*., 1997). In advanced HIV, cryptosporidiosis is associated with pneumatosis cystoides intestinalis in which cysts containing gas that occur in the wall of the gut can rupture and cause pneumoretroperitoneum and pneumomediastinum (Hunter *et al.,* 2002).

Cryptosporidiosis in bone marrow and solid organ transplant patients has also been studied. Hunter and Nicholas (2002) have reviewed the severe disease seen in bone marrow transplant patients and found that the severity of cryptosporidiosis depended on the underlying disease for which the transplant was performed. In solid organ recipients and cancer patients (other than with haematological malignancies), cryptosporidiosis was not the problem it is with haematological malignancies (Hunter and Nicholas, 2002).

1.1.7.5 Diagnosis

Different specimens can be examined for *Cryptosporidium* including stool, intestinal and gastric biopsies, bile, sputum or bronchioalveolar lavage and antral washout.

1.1.7.5.1 Stool examination

Detection of oocysts in stool sample remains the easiest way to diagnose the infection. However a negative result in microbiological examination of a single stool sample cannot exclude cryptosporidiosis since oocysts may fall below detectable numbers even during symptomatic infection (Jokipii and Jokipii, 1986). Therefore it was suggested that collection of samples at different times is the best way for diagnosis (Weber *et al.,* 1991) and the triple faeces test approach (based on 3 consecutive daily samples) significantly increased detection rates (Van Gool *et al.,* 2003).

The usual and easiest method for detection of oocysts in the stool is by acid-fast or fluorescent (auramine-phenol) staining and microscopy. This can often detect the organism in high numbers.

Direct and indirect immunofluorescence microscopy for oocysts and ELISA for oocyst wall antigen are also used. Anti-cryptosporidial antibodies can be used in immunomagnetic separation followed by immunofluorescent microscopy to detect

as few as two oocysts per gram stool and may be reliable in managing severely affected patients regardless of faecal consistency (Robinson *et al.,* 2008). In experimental studies flow cytometry has been used to quantify oocysts after isolation from stool samples (Moss and Arrowood, 2001).

1.1.7.5.2 Histological examination

Small bowel or gastric biopsies are occasionally used in diagnosis especially in patients with persistent diarrhoea or in patients with profound T cell immune deficiency when the stool sample is negative. The histopathology examination will show variation in parasite burden and degree of injury from area to area and between patients. In some infections villous atrophy and crypt hyperplasia with mixed inflammatory cell infiltration of lamina propria can be seen under light microscopy (Lumadue *et al*., 1998).

1.1.7.5.3 Polymerase chain reaction (PCR)

This is a very sensitive technique that is useful for testing stool when a low number of oocysts is suspected (McLauchlin *et al.,* 2003) and it is the only method that gives information about the different species and genotypes. PCR can be applied to stool, tissues and other specimen types such as bile taken during endoscopic examination. PCR amplification has targeted the genes encoding the major wall protein of the oocyst, the small unit of rRNA, β-tubulin, TRAP-C1, TRAP-C2, ITS1, dihydrofolate reductase and non-coding satellite DNA sequences. Further treatments (eg using DNA restriction enzymes) can be employed to detect different *Cryptosporidium* species (Higgins *et al.,* 2001).

1.1.7.5.4 Radiological examination

Radiological tests including abdominal ultrasound scan (US) and computerised tomography (CT) scan have been used in patients with extra-intestinal

cryptosporidiosis. US examination of a patient with biliary disease has shown a generalised dilatation of the bile duct and gall bladder (Chen *et al.,* 2002). If the US examination is normal and there is a high suspicion of biliary involvement, endoscopic retrograde cholangiopancreatography **(**ERCP) should be considered (Chen *et al*., 2002).

1.1.7.6Management

Non-specific supportive treatment, including oral or intravenous rehydration, electrolyte and nutritional supplementation are currently the most essential treatments to manage the disease.

1.1.7.6.1 Drug therapy

Drug treatment of cryptosporidiosis falls commonly into three groups: antimicrobial therapy, immunotherapy and symptomatic anti-diarrhoeal treatment. Drugs from more than one group have been used in combination.

1.1.7.6.1.1 Antimicrobial therapy

Cryptosporidium is known for its resistance to antimicrobial therapy; the reason for this is not understood but could be related to the unique epicellular localization of the parasite in the host cell.

Nitazoxanide and paromomycin are the only drugs which when examined in controlled clinical trials showed some efficacy in the resolution of the infection (Rossignol *et al*., 2009). Nitazoxanide, the most efficacious compound, is approved by the United States Food and Drug Administration for use in immunocomeptent patients older than 1 year and is available by regular prescription. In the UK, nitazoxanide is not licensed but is available on a named patient basis. Amadi *et al.* showed that in a placebo controlled trial of Zambian HIV positive and negative children infected with *Cryptosporidium,* treatment with 100mg nitazoxanide twice daily for three days resulted in significant improvement in diarrhoea and parasite

clearance among many HIV negative patients. In the HIV positive group no benefit was found after the primary course of treatment but after a second course of therapy 77% of patients showed some response (Amadi *et al.,* 2002). A double-blind placebo controlled study in Mexican HIV positive patients reported that treatment with higher doses of nitazoxanide was significantly effective for parasite clearance in comparison to the placebo group. Parasite shedding and diarrhoea resolved in patients with a CD4 cell count higher than 50 but not in those with lower CD4 cell counts (Rossignol *et al*., 1998).

Paromomycin was one of the first drugs tested for treating cryptosporidial diarrhoea. Its effect was investigated in several small and mostly uncontrolled studies, and it usually had modest activity against *Cryptosporidium* (Griffiths *et al*.,1998).

Other drugs proposed for cryptosporidiosis such as sinfungin and metronidazole were only tested in laboratory neonatal rats but there is no evidence of effectiveness in treating human disease (Rossignol, 2010). Some drugs including spiramycin, clarithromycin, octerotide acetate, atovaquone, letrazuril and lasalocid were tested in a limited number of patients with AIDS-related cryptosporidiosis and failed to show antidiarrhoeal or antiparasitic activity (Zardi *et al.,* 2005). Limited clinical trials studied the effect of azithromycin and roxithromycin in the treatment of diarrhoea in AIDS. Short or prolonged term of treatment with azithromycin did not affect oocyst shedding (Kadappu *et al*., 2002). An open-label trial studied the effect of roxithromycin in 26 AIDS patients. About 68% of treated patients were cured and 6% were considered improved while one patient failed to improve. However, due to the lack of a control group and due to the variable-cyclical nature of the disease these findings were considered unreliable (Uip *et al.,* 1998).

1.1.7.6.1.2 Antiretroviral therapy

Highly active antiretroviral therapy (HAART) is the best choice of treatment for patients with HIV-related disease. It controls viremia and increases CD4 cell

numbers. *In vitro*, protease inhibitors used in HAART (e.g. nelfinavir, indinavir, ritonavir) reduced *C. parvum* host cell invasion and parasite development and this inhibition was enhanced when used in combination with paromomycin (Hommer *et al.,* 2003; Schmidt *et al.,* 2001). These drugs restore CD4 cells and lead to increase in the expression of interferon-γ, IL-15, IL-4 even in those with modest degrees of immunoreconstitution.

HAART has also been attributed to recovery of cryptosporidiosis in non-HIV immunocompromised patients such as in primary immunodeficiency, organ transplantation, cancer and malnutrition for which this therapy is not indicated (Abdo *et al*., 2003).

1.1.7.6.2 Passive immunotherapy

Results from studies using orally administered bovine colostrum containing antibodies against *Cryptosporidium* are contradictory and no controlled clinical data are published (Rossignol, 2009). Some patients responded well to colostrum from cows immunised with *C. parvum* oocyst antigen plus adjuvant but others showed no benefit. A similar type of study investigating the effect of neutralizing monoclonal antibodies as therapy against persistent *C. parvum* infection in adult IFN-$\gamma^{-/-}$ SCID mice showed a significant reduction of infection level in treated mice without eradicating the parasites. (Riggs *et al.,* 2002).

1.1.7.6.3 Probiotics

Probiotics have been used successfully in the treatment of acute diarrhoea caused by different pathogens. Few studies have investigated the effect of probiotics on *Cryptosporidium* infection. Pickerd and Tuthill (2004) reported a single case of a beneficial effect of *Lactobacillus osporGptG* in the treatment of prolonged cryptosporidiosis in a patient with coeliac disease. A clinical trial in Peru, however,

found no beneficial effect of milk formula containing *Lactobacillus GG* in infants with acute diarrhoea with different causes including *C .parvum* (Salazar-Lindo *et al.,* 2004).

Other results from studies with immunocompromised animals were contradictory. A beneficial effect of probiotics on *Cryptosporidium* infection has been shown, but the mechanism(s) for this effect was not established (Alak *et al.,* 1997, 1999).Guitard *et al*. (2006) did not find a significant effect for a *L. casei* containing mixture milk formula in eradicating the parasite in suckling rats (Guitard *et al.,* 2006).

1.1.7.7 Prevention and control

Since treatment modalities for cryptosporidiosisare limited, prevention and risk reduction are important in the control of the disease. Cryptosporidiosisis highly infectious in person-person transmission and therefore meticulous personal hygiene is required. Summarised guidelines for person-person hygiene (Anon, 2004) include frequent hand washing particularly when caring for a person with diarrhoea, proper disposal of excreta and washing of soiled materials such as clothing and bedding.

People with cryptosporidiosisshould not attend their work place, school or other institutions for 48h after the last diarrhoeal episode, particularly food handlers and staff of healthcare facilities (Anon, 2004). Exclusion from using swimming pools for 2 weeks after diarrhoea has stopped is preferred. General precautions against the infection include hand washing prior to eating or preparing food and after contact with animals and washing of fruits and vegetables prior to consumption. Suitable hand washing facilities should be provided at open farms where transmission is common.

As contaminated water remains the main source of human infection, special attention should be paid to boiling water or careful filtration and as the parasites are chloride resistant, disinfection techniques using ultraviolet light or ozone at water treatment contents might be helpful (Ramirez *et al*., 2004). Immunocompromised

patients are also advised to boil all drinking water to reduce the risk of infection (Chief Medical Officer update, 1999).

Means that reduce transmission between animals and between animals and humans should also be encouraged. People who are at high risk of infection should avoid contact with animals with diarrhoea or at least animals should be examined before allowing contact with humans (Juranek, 1995).

In the UK, cryptosporidiosis is notifiable only where it is believed infection is food or water-borne, while in the US, it is a nationally notifiable disease (Davies and Chalmers, 2009).

SECTION II: HOST IMMUNE RESPONSES TO *CRYPTOSPORIDIUM*

1.2.1 Introduction

Although many studies have examined immunity to *Cryptosporidium*, particularly *C. parvum,* the detailed protective immune responses against this parasite are yet to be understood.

A variety of innate immunity components and responses may partially control the early parasite infection and produce signals for T cell activation such as IFN-γ from NK cells, certain chemokines, defensins and pro-inflammatory cytokines. Studies support an important T_H1 pathway in the ultimate control and elimination of infection. However, an additional involvement of IL-4 in protection could also indicate a T_H2 response involvement.

Mice and other animals generally develop “natural resistance” to *C. parvum* infection before weaning (Sherwood *et al.,* 1982). To circumvent this natural resistance, neonatal and immunocompromised mice are often used and have been found to be suitable models to study immunity to *C. parvum* (Tzipori 1988). Studies have also involved the gastric parasite *C. muris* which does grow readily in adult mice but this parasite is non-pathogenic. Human and bovine studies to investigate immunological studies (mostly against *C. parvum*) are limited, but most of the results have been in agreement with those obtained with murine models (McDonald, 2007).

1.2.2 Innate Immunity

Innate immunity provides the first line of host defence against pathogens and may limit development of infection via different antimicrobial killing mechanisms until the more potent adaptive immune response takes effect. Components of the innate immune system include the inflammatory cells such as NK cells, neutrophils, macrophages and eosinophils as well as the non-immune cells including intestinal epithelial cells (enterocytes). Products released by innate immune cells including chemokines, cytokines, complement factors and antimicrobial peptides are important directly or indirectly in microbicidal mechanisms (Delves and Roitt, 2000).

1.2.2.1 The role of intestinal flora

Some studies have suggested a protective role of the normal intestinal flora against cryptosporidial infection. The onset of refractoriness of healthy immunocompetent mice to *C. parvum* infection coincides with the established colonization of the gut by resident bacteria. Harp *et al.* found that germ-free mice were more susceptible to infection than conventional mice (Harp *et al.,*1988). This could be due tothe physical presence of a flora in the intestine resulting in competition for receptor sites, production of anticryptosporidial agents, and stimulation of gut motility could all be involved in blocking colonization by the parasite (Harp *et al.,*1988). Additionally the antigenic stimulation provided by the gut flora could perhaps be responsible for the activation of components of the immune system mediating resistance to *C. parvum*(Harp *et al.,*1988). Few studies have investigated the effect of probiotics on *Cryptosporidium* infection. Pickerd and Tuthill (2004) reported a single case of a beneficial effect of *Lactobacillus osporGptG* in the treatment of prolonged cryptosporidiosis in a patient with coeliac disease. A clinical trial in Peru, however, found no beneficial effect of milk

formula containing *Lactobacillus GG* in infants with acute diarrhoea with different causes including *C. parvum* (Salazar-Lindo *et al.,* 2004).

1.2.2.2 Epithelial cells

Epithelial cells are the only known cells that can be infected by *Cryptosporidiumin vivo* and infection by pathogenic species predominantly occurs in the intestinal epithelial cell (enterocytes) (Sears and Guerrant, 1994). Enterocytes produce inflammatory molecules including chemokines, express toll-like receptors (TLRs) which act as sensors of infection and stimulate immune mechanisms for microbial killing (Zhou *et al.,* 2012).

The initial interaction between *C. parvum* and intestinal epithelial cells activates NF-κB, an important transcription factor for different inflammatory signalling pathways. The initial stimulus for NF-kB activation is unclear but parasite products might interact with TLR molecules.

Originally, it was shown that *C. parvum* infection of enterocytes would induce apoptosis of infected cells as an attempt to limit spread of infection (Chen *et al.,*1998). In infected cell monolayers, uninfected cells also underwent apoptosis due in part to secretion of FasL by infected cells (Chen *et al.,* 1999) However, later work indicated that the parasite is able to oppose this effect by the activation of NF-kB that induces a number of apoptosis inhibitors (Chen *et al.,* 2001).

In a recent study with *C. parvum* infection of piglets, Foster *et al* observed that there was extensive shedding of infected cells associated with apoptosis from the piglet epithelium but this occurred mostly at the villous tips, where epithelial cells are normally shed. A key step in initiating apoptosis, the cleavage of caspase-3, did occur but the enzyme function was prevented by the binding of an apoptotic inhibitor XIAP and proteasome activity. At the villus tips, NF-êB activation was less pronounced in cells being shed into the gut lumenand most of these cells were apoptotic. This indicated

that suppression of apoptosis except at the villus tips allows elimination of infected cells in a controlled manner that minimised damage to the intestinal epithelial barrier (Foster *et al.,* 2012).

Infection of epithelial cell lines with *C. parvum* activated the expression of proinflammatory NF-kB-dependant chemokines including IL-8, GRO-α, RANTES, MCP-1 and MIP-2α (Laurent *et al.,* 1997; Lacroix-Lamandé *et al.,* 2002). The exact role of these chemokines in immunity is not known, but they are important initiators of the inflammatory response. Lacroix-Lamandé *et al*, have also shown that a lack of the chemokine receptor CCR5 had an impact in the early stage of *C. parvum* infection in neonatal mice as mice lacking CCR5 had a higher parasite burden early during the course of infection but this receptor was dispensable for subsequent parasite elimination (Lacroix-Lamandé *et al.,* 2008).

Importantly, *C. parvum*-infected intestinal epithelial cell lines were also found to produce a variety of dendritic cell-attracting molecules (CCL2, CCL3, CCL4, CCL5, CCL7 and CCL20) which enhance migration of these cells to the infection site (Auray *et al*., 2007).

C. parvum infection of epithelial cells also induced prostaglandins which may have different effects such as modulation of T cell responses, decreasing inflammation and increasing production of mucin by goblet cells that would protect the epithelium (Laurent *et al.,* 1998).

The role of NO in the innate immunity to *C. parvum* was also described. Piglets infected by *C. parvum* were shown to have increased NF-êB-dependent intestinal expression of iNOS leading to NO production (Gookin *et al.,* 2006). The iNOS expression was mainly happened in the epithelium and treatment with an iNOS inhibitor increased parasite reproduction suggesting that the iNOS stimulation is protective. This activity is at least partly an innate immune responses as it is initiated soon after infection (Gookin *et al.,*

2006). In a recent study Zhou *et al.* showed that, a significant increase in NO production was detected in epithelial cell lines following *C. parvum* infection, this was regulated by iNOS as confirmed by a significant increase of iNOS protein and mRNA expression in those cells (Zhou *et al*., 2012).

1.2.2.2.1 Toll-Like Receptors

Toll-like receptors (TLRs) are a family of pattern recognition receptors which recognise distinct molecular patterns associated with microbial pathogens (Takeda *et al*., 2003). These receptors constitute the first line of defence against many pathogens and play a crucial role in the function of the innate immune system by activating NF-kB and other signalling pathways to produce inflammatory cytokines and chemokines. Ten human and twelve murine TLRs have been identified, TLR1 to TLR10 in humans, and TLR1 to TLR9, TLR11, TLR12 and TLR13 in mice. For example, TLR2 is essential for the recognition of a variety of pathogen associated molecular patterns (PAMPs) from Gram-positive bacteria, including bacterial lipoproteins, lipomannans and lipoteichoic acids. TLR4 is activated by lipopolysaccharide. TLR9 is required for response to unmethylated CpG DNA. (Takeda and Akira, 2005). Signal transduction by members of this family except TLR3 is initiated by an adaptor molecule, MyD88 (Akira and Takeda, 2004). Intestinal epithelial cells are capable of expressing most TLRs (Gewirtz, 2003) where TLRs play dual roles in protecting the mucosal surface by helping to maintain homeostasis and promoting inflammation following mucosal injury(Santaolalla and Abreu, 2012).

Evidence for the role of TLRs in *C. parvum* infection has been reported. MyD88 has been shown to be activated during *C. parvum* infection (Chen *et al.,* 2001). In another study, Chen *et al.* have shown that infection of a bile duct epithelial cell line with *C.*

parvum induces the recruitment of TLR2 and TLR4 and not other TLRs to the parasite attachment site on the cell membrane and this resulted in activation of IRAK and phosphorylationof the MAPK p-38. Moreover, it was found that transfection of the cells with dominant-negative TLR2, TLR4 or MyD88 mutants or treatment of cells with interference RNA to deplete TLR2 or TLR4 inhibited NF-kB activation by the parasite. This study also showed that deficiency of MyD88 increased susceptiblyto *C. parvum* infection (Chen *et al.,* 2005). Another study suggested that the MyD88 pathway plays a role in immunity to *C. parvum* as the infection level observed in juvenile MyD88$^{-/-}$ mice was significantly higher than the level in wild-type mice (Rogers *et al*.,2006). In agreement with the significance of TLR4 for countering infection *in vitro*, compared with control animals TLR4$^{-/-}$ mice took longer to clear infection from the intestine and bile ducts and had an altered and enhanced hepatic inflammatory response (O'Hara *et al.,* 2011).

A cellular micro-RNA, let-7i that down-regulates TLR4 expression, had expression in biliary epithelial cells infected by *C. parvum* so that TLR4 expression was increased and so contributed to the epithelial resistance against *C. parvum* infection (Chen *et al*., 2007). Supporting the role of TLR4, Zhou *et al.* demonstrated that a significant increase in NO production was detected in TLR4-responsive epithelial cell lines following *C. parvum* infection, whereas no NO was detected in Caco-2 cells which do not express TLR4 (Suzuki *et al.,* 2003). They also observed a significant increase of iNOS protein in biliary epithelial cells of infected mice compared to no increase in the TLR4 deficient mice (Zhou *et al.,* 2012). The mechanism of the increase expression of iNOS was dependent on the suppression of KH-type splicing regulatory protein (KSRP) through the up-regulation of a NF-κB responsive microRNA (miR27b) gene (Zhou *et al.,* 2012). Interestingly, this iNOS expression is completely independent of IFN-γ, the classical inducer required for expression of this enzyme in macrophages.

In an *in vitro* study of infection of human biliary cells by O'Hara *et al.* it was demonstrated that an immunodeficiency virus type 1 (HIV-1) derived peptide (Tat) inhibited the ability of the these epithelial cells to express TLR4 upon infection with *C. parvum* and more parasites were found in Tat-treated cells than in control cells 48 h after infection. It was therefore suggested that these findings may partly explain the increased susceptibility of HIV-infected individuals to biliary cryptosporidiosis (O'Hara *et al.,* 2009). It is not known whether these latter findings with biliary epithelial cells apply to enterocytes.

With regard to TLR9, Barrier *et al.* found that treatment of neonatal mice with a synthetic oligodeoxynucleotide CpG that is a ligand for TLR9 stimulated strong resistance against parasite reproduction (Barrier *et al.,* 2006). However, in similar experiments with adult malnourished mice which are susceptible to infection CpG treatment reduced the parasite load only by a modest degree (Costa *et al.,* 2012).The reason for differences between degrees of resistance to infection in these two studies could be due to the nature of infection models employed or it is possible TLR9 stimulation is more readily achieved in the neonatal mouse.

Malnutrition in human infants is often associated with poor control of cryptosporidial infection (Gendrel*et al.,* 2003). A recent *in vivo* study by Costa *et al.* studied the effect of malnourishment on infection with *C. parvum* in mice*.* It was found that adult malnourished C57BL/6 mice developed a higher level of infection as well as a significant reduction in the villous height–crypt depth ratio in the ileum in comparison to well-nourished infected mice. This was associated with a significant depression of expression of TLR2 and TLR4 mRNA in the ileum. Furthermore, malnutrition and infection resulted in reduced ileal TNF-α and IFN-γ levels compared with infected controls. However, no difference was observed in TLR9 expression between infected

(nourished or malnourished) and uninfected mice. These observations concluded that in the weaned animal, malnutrition intensifies cryptosporidial infection that may be explained by depressed TLR2 and TLR4 expression leading to lower expression levels of Th1 cytokines (Costa *et al*., 2011). The measurement of TLR expression was by qPCR using whole tissue, however, so the cells affected were not known. Neverthess, these observations supports the role of TLR/NF-κB mediated innate immune responses by epithelial cells might be critical for the host defense to *C. parvum*.

1.2.2.2.2 Antimicrobial peptides

The enterocyte immunological activity also extends to antimicrobial peptide production, such as defensins. Increased enteric β-defensin expression was first shown in calves infected with *C. parvum* (Tarver *et al*., 1998). Zaalouk *et al.* demonstrated that *in vitroC. parvum* infection of murine and human enterocyte cell lines downregulated the constitutive development of β-defensin-1 while an upregulation of human β-defensin-2 was observed. Human β-defensin-1 and -2 killed many sporozoites as measured by flow cytometry and caused reduced development after addition to an enterocyte cell line. *In vivo,* murine intestinal β-defensin-1 expression was also eliminated after infection with the parasite. (Zaalouk *et al.,* 2004).

Epithelial cell line constitutive expression of IL-18 which has been reported to be increased with *C. parvum* infection *in vitro* (McDonald *et al.,* 2006) and the exogenous treatment of enterocytes with this cytokine reduced parasite development. This was found to be associated with increased expression of antimicrobial peptides LL-37 and β-defensin-2 (McDonald *et al.,* 2006).

1.2.2.2.3 Complement

Enterocytes are able to produce some complement components (Moon *et al*., 1997). So, another possible mechanism of *C. parvum* inactivation in the gut wall is the binding of the parasite sporozoites or merozoites to complement. Activation of the complement cascade classically can be initiated by two mechanisms which lead to enzyme cleavage of C4 and C2 to form C3 convertase. This may be dependent on the presence of C1 or the mannose binding lectin (MBL) which is a calcium-dependent protein that plays a role in the innate immune response by binding to carbohydrates on the surface of a range of pathogens (viruses, bacteria, fungi, protozoa) where it can activate the complement system or act directly as an opsonin (Koch *et al*, 2001). HIV patients with homozygous mutations in the MBL gene were more susceptible to *C. parvum* infection and serum deficiency of MBL in young children correlated with an increased incidence of cryptosporidiosis (Kelly *et al.*, 2000; Kirkpatrick *et al*., 2006). Both MBL and C4 were found to adhere to the parasite sporozoites indicating that MBL may directly block parasite attachment to the epithelial cells or activate the complement complex (Kelly *et al.,* 2000). A study by Petry *et al.* demonstrated that *C. parvum* can activate both the classical and lectin pathways, leading to the deposition of C3b on the parasite (Petry *et al.,* 2008). Furthermore, human MBL bound to sporozoites as well as intact oocysts and empty oocysts wall. However, a comparison of the level of infection in complement factor-depleted ($C1qA^{-/-}$), MBL -depleted ($MBL\text{-}A/C^{-/-}$) and $IL\text{-}12^{-/-}$ mice, showed that only $IL\text{-}12^{-/-}$ mice developed severe infection. Additionally, some parasite development was detected in MBL- $A/C^{-/-}$ but not in $C1qA^{-/-}$ or wild type mice. Taken overall, the results of studies suggest that complement may play a role in immunity to *C. parvum* only when the immune system is substantially weakened.

1.2.2.3 Role of immune cells in innate immunity to *C. parvum*

Innate immune cells including NK cells, macrophages and neutrophils can play important roles in immunity to infection. They produce cytokines which might either directly affect the parasite reproduction or stimulate other cells to control infection and can directly kill microbial pathogens by cytolysis of infected cells or direct antimicrobial killing mechanisms.

1.2.2.3.1 Neutrophils and Macrophages

A study of *C. parvum* infection of piglets failed to show any effect on the parasite reproduction in animals treated with antibody to deplete neutrophils in comparison to the control animals (Zadrozny *et al.,* 2006). However more recent work by Takeuchi *et al.* has demonstrated an important role for neutrophils and macrophages in acute *C. parvum* infection. In this study a comparison of *C. parvum* infection was made in SCID beige (SCIDbg) (lack T, B, NK cell cytotoxicity and to a degree neutrophil exocytosis), and SCIDbgMN mice (SCIDbg mice depleted of functional macrophages and neutrophils). SCIDbgMN mice were created from SCIDbg mice after whole body X irradiation followed by treatment with carrageenan and anti-Ly6G monoclonal antibody (Takeuchi *et al*., 2008). In infected SCIDbg mice oocyst excretion was first detected on day 18 and increased gradually but all mice survived infection. In contrast, in SCIDbgMN mice oocyst shedding began within 3 days of infection and reached a peaked at day 4 and mice died within 16 days. However, the resistance of SCIDbgMN mice to *C. parvum* infection was completely regained to the level shown by SCIDbg mice after inoculation of these mice with peritoneal macrophages from *C. parvum*-infected SCIDbg mice (CP-macrophages) identified as conventionally activated M1 macrophages or resident macrophages plus CP-neutrophils (neutrophils from infected

SCIDbg mice). Results from the same study also indicated that CP-neutrophils were a source of IFN-γ that is required for the activation of resident macrophages to M1 macrophages. Therefore, M1 macrophages may act as the final effector cells in host resistance against acute *C. parvum* infection (Takeuchi *et al*., 2008). The protective role of macrophages in chronic *C. parvum* infection of $Rag2^{-/-}\gamma c^{-/-}$ mice (lack T, B and NK cells) was also demonestrated. These mice surprisingly have a low level of chronic infection for several weeks before the infection intensifies leading to death. Depletion of macrophages while the infection level was low resulted in a sharp increase in the level of oocyst production and death within days in comparison to the untreated infected mice. Normally in $Rag2^{-/-}\gamma c^{-/-}$ mice intestinal levels of IFN-γ increased as infection eventually worsened but no rise in IFN-γ mRNA was detected in macrophage-depleted mice (Choudhry *et al*., 2012). Together, these findings indicate an important role for macrophages in the protective innate immune response to *C. parvum*, at least in the absence of lymphocytes, and imply macrophages are a key source of IFN-γ. It is not known, however, whether immunity would be so dependent on macrophages in the presence of lymphocytes.

1.2.2.3.2 Natural Killer Cells

Natural killer (NK) cells are an important cellular component of the innate immune response against intracellular infection and also tumour cells (Lodoen and Lanier, 2006). They can be cytotoxic and may also produce inflammatory cytokines such as IFN-γ and TNF-α. The role of NK cells in innate immunity will be discussed in detail in Chapter 4 of this thesis.

1.2.2.3.3 Dendritic Cells

The protective role of dendritic cells against cryptosporidium has not been well investigated. Auray *et al,*indicted that dendritic cell-attracting chemokines are produced by epithelial cells in response to *C. parvum* (Auray *et al.,* 2007). Furthermore, a recent study by Bedi and Mead has shown that *C. parvum* antigens induced DC activation as indicated by upregulation of the maturation marker CD209. It also induced the production of cytokines including IL-12, IL-2, IL-1 and IL-6. In the same investigation soluble sporozoite antigen or live sporozoites activated dendritic cells derived from human peripheral blood cells to produce IL-12 (Bedi and Mead, 2012).

1.2.2.4 The role of IFN-γ and other cytokines in innate immune responses to *C. parvum*

1.2.2.4.1The role of IFN-γ

Studies with adult SCID mice have shown that these mice develop chronic infection which is normally for some weeks mild in nature but becomes progressive and then fatal (McDonald *et al.,*1992). IFN-γ has been identified as a key cytokine in protective innate and adaptive immune responses to *C. parvum* infection. Numerous studies have addressed the role of this cytokine in innate immunity. A study with SCID mice with a targeted IFN-γ gene mutation indicated a protective role for this cytokine as the IFN-γ-deficient mice developed a more intense infection than control SCID mice (Hayward *et al.*, 2000). Similarly, treatment of athymic nude mice, SCID mice, or mice depleted of $CD4^+$ T cells with a neutralizing antibody to IFN-γ increased the infection level and shortened the period before morbidity would occur (Ungar *et al.,* 1991; McDonald and Bancroft, 1994). Hence, IFN-γ undoubtedly plays a major role in the early control of infection and innate immunity.

In an *in vitro* study by Pollok *et al.* (2001) it was shown that treatment of cultured enterocyte cell lines with exogenous IFN-γ decreased *C. parvum* reproduction. Two mechanisms of inhibition of parasite development were identified: a reduction of cell invasion by sporozoites and, more importantly, depletion of cellular Fe^{2+} that would be important for intracellular parasite growth (Pollok *et al.,* 2001). Brandacher *et al.* have reported that IFN-γ induces enterocytes to express indoleamine 2,3 dioxygenase (IDO) that catabolises tryptophan required for microbial growth (Brandacher *et al.,* 2006).This, however was not the anti-parasitic mechanism of IFN-γ in *C. parvum* and interestingly it was recently found in this laboratory that *C. parvum* infection inhibited IDO expression (Choudhry *et al.,* 2009). Infection depleted expression of STAT1, the key transcription factor in the IFN-γ signalling pathway, which may underline the importance for this cytokine in the activation of enterocytes as part of immunity to infection.

The inhibitory effect of IFN-γ on parasite reproduction *in vitro* was found to be inhibited by the anti-inflammatory cytokine TGF-β, but not IL-10. Significantly, IL-4 that classically inhibits IFN-γ functions was found to work synergistically with IFN-γ and increased parasite killing activity when used together with low concentrations of IFN-γ (Lean *et al.,* 2003). The mechanism underlying this synergistic activity was not established but was not related to expression or phosphorylation of STAT1.

1.2.2.4.2 The role of other cytokines

Other cytokines have also been shown to play roles in innate immunity to *C. parvum*.

IL-12: This is an important cytokine in innate immunity as it activates NK cells to produce IFN-γ (Lieberman and Hunter, 2002). The treatment of neonatal BALB/c mice with an antibody for IL-12 exacerbated *C. parvum* infection while exogenous IL-12 treatment prior to oocyst inoculation prevented the development of infection. This

protective effect was completely blocked by anti IFN-γ antibody (Urban *et al.,* 1996). Furthermore, intestinal epithelial cell invasion and/or early intracellular development of *C. parvum* was inhibited by treatment of mice with IL-12 24 h before oocyst inoculation (Urban *et al.,* 1996)

TNF-α: TNF-αis another important innate immune component produced by macrophages and lymphocytes and can stimulate NK cells to produce IFN-γ (Hunter *et al*., 1994). High levels of TNF-α were expressed in *Cryptosporidium* infected intestinal tissue samples of both mice and humans (Lacroix *et al.,* 2001; Robinson *et al.,* 2001; Ehigiator *et al.,* 2005). In a study by Lacroix *et al.* 2001, the failure of C57BL/6 IFN-$\gamma^{-/-}$ mice to control infection was associated with poor intestinal expression of TNF-α in comparison to the infected wild-type mice. The same study also showed that the treatment of these mice with TNF-α reduced parasite development (Lacroix *et al.* 2001). In an *in vitro* study, TNF-α inhibited parasite development in cultured human and mouse enterocyte cell lines (Pollok *et al.,* 2001; Lean *et al.,* 2006) and it was shown that the mechanism of this inhibitory action was by reducing the parasite invasion of the enterocytes (Lean *et al.,* 2006). However, in the same study by Lean *et al.* it was found that TNF-α was not required for the control of *C. parvum* infection in neonatal mice as no differences in the level of infection were detected between TNF-$\alpha^{-/-}$ mice and wild type mice (Lean *et al.,* 2006). This supported earlier work by two groups which indicated that TNF-α activity was not necessary for the control of infection as the treatment of SCID mice with anti-TNF-α neutralizing antibodies did not cause an increase in *C. parvum* reproduction (McDonald *et al.,* 1992; Chen *et al.,* 1993). Together, these findings suggest that TNF-α may not play a major role in resistance to cryptosporidiosis or has a role that is replaceable.

IL-1: IL-1 has also been found to be up-regulated during infection of neonatal mice and this was independent of TNF-α expression (Lean *et al.,* 2006). The cytokine also inhibited parasite reproduction in epithelial cells (Pollok *et al.,* 2001). Both TNF-α and IL-1 are stimulators of prostaglandins and it was previously demonstrated that *C. parvum* infection directlystimulated human epithelial cell line HCT-8 to produce prostaglandin H synthase 2 (PGHS2), prostaglandins PGE_2 and PGF_{2a} (Laurent *et al.*,1998). The possible functions of these prostaglandins may include: regulation of epithelial Cl^- levels along with fluid secretion (Argenzio*et al.*, 1993) and increased mucin expression which may protect cells from infection (Hill *et al.,* 1991). PGE_2 has also been shown to down-regulate inflammatory cytokine production by macrophages (Knudsen*et al.,* 1986).

IL-15: IL-15 is a product of macrophages, dendritic cells and epithelial cells and is an important activator of NK cells and T cells to produce IFN-γ as well as increasing NK cell cytotoxicity. It also plays an important role in NK cell development and homeostasis of NK cells (Kim *et al*., 2008). In *C. parvum* infection of immunocompetent volunteers those with symptomatic infection failed to express IFN-γ in the intestine, but IL-15 was usually detected (Robinson *et al.,* 2001), indicating that IFN-γ-independent protective pathways may be possible. Another study with IL-12 deficient C57BL/6 mice, however, showed that despite heavy infection with the parasite the mice were able to recover and expressed both IFN-γ and IL-15 during early infection (Ehigiator *et al.,* 2005).

IL18: IL-18 is a proinflammatory cytokine that like IL-15 is produced by epithelial cells, macrophages and dendritic cells (Iannello *et al.,* 2009). It acts with other cytokines such as IL-12 or IL-15 to stimulate IFN-γ production (Okamura *et al.,* 1998). In an *in vitro* study, infection of human enterocytes cell lines enhanced IL-18 mRNA expression and exogenous IL-18 treatment of these cell lines reduced parasite reproduction in these

cell lines (mentioned above), suggesting a possible protective role for this cytokine against *C. parvum* infection(McDonald *et al.,* 2006). The role of IL-18 in innate immunity to this parasite was recently confirmed by a study in this laboratory by Choudhry *et al. C. parvum*-infected adult immunocompromised alymphocytic $Rag2^{-/-}\gamma c^{-/-}$ mice expressed high levels of intestinal IL-18 mRNA and caspase-1 (which is important for production of mature IL-18 protein from pro-IL-18) (Lannello *et al.,* 2009). In addition, the treatment of these mice with anti-IL-18-neutralizing antibodies impaired resistance to infection and this was associated with a decreased level of intestinal IFN-γ expression (Choudhry *et al.,* 2012). The same study demonstrated that the murine intestinal epithelial cell line CMT-93 produced IL-18 following *C. parvum* infection and a combination of IFN-γ and infection of these cells resulted in an even higher level of IL-18 expression, while IFN-γ on its own had no effect on IL-18 production.Furthermore, IL-18 together with IL-12, but not any of several other proinflammatory cytokines, stimulated cultured peritoneal macrophages to produce IFN-γ. IL-18 therefore plays a protective innate immunological role against *C. parvum* infection and one possible mechanism is by promoting IFN-γ production by macrophages (Choudhry *et al.,* 2012).

IL-4: IL-4 is an important cytokine in driving the Th2 responses and studies of the role of this cytokine in the immunity to cryptosporidiosis are contradictory. However, McDonald *et al.* have shown that IL-4 mRNA became detectable in intestinal samples of neonatal BALB/c mice at 24h after infection with *C. parvum.* Furthermore, increased oocyst shedding was observed in neonatal mice treated with anti-IL-4 neutralizing antibodies in comparison to the control mice and this protective role of IL-4 was IFN-γ-dependant (McDonald *et al.*, 2004). *In vitro,* IL-4 was also found to act synergistically with IFN-γ to induce antimicrobial killing of the parasite by enterocytes (Lean *et al.,* 2003).

Both IL-13 and IL-4 employ the same receptor (IL-4Rα) and have overlapping functions (Wynn, 2003). A study by McDonald *et al,* showed that neonatal BALB/c IL-4Rα$^{-/-}$ developed more intense infection that wild-type mice (McDonald *et al*., 2004), but unlike IL-4, IL-13 did not increase IFN-γ inhibitory function against *C. parvum* in cultured enterocyte (Lean *et al.,* 2003).

IL-6: Elevated levels of and IL-6 were detected in neonatal IFN-γ knockout (C57BL/6-GKO) mice after *C. parvum* infection (Lacroix *et al*., 2001) that failed to control infection and the cytokine was shown to reduce development of this parasite *in vitro* (V. McDonald, unpublished data). However, increased IL-6 expression in the intestine was associated with the inability of IFN-γ$^{-/-}$ mice to control infection (Lacroix et al., 2001) and also with hepatic necrosis in TLR-4$^{-/-}$ mice (O' Hara et al., 2011).

Type I IFNs:The central role of type I IFNs as inducers of antiviral host responses is well established and a part from strong antiviral activities, type I IFNs were found to play roles in immunity to non-viral microbial organisms including protozoa (Bodgan *et al.,* 2004). The role of type I IFN in immunity to *Cryptosporidium* has been unknown and this will be discussed in detail in Chapter 3 of this thesis.

1.2.2.5 Immunity to *C. parvum* infection in neonatal mice

As stated previously, only very mild infections of *C. parvum* are obtained in immunocompetentadult animals, including mice (Sherwood *et al.,* 1982). Immunological activity is obviously one important factor in resistance as adult mice lacking T cells or T cells and B cells develop chronic infections that are often eventually fatal (McDonlad *et al.,* 1992). Adaptive immune mechanisms against *C. parvum* have often been investigated using adult mice of immunocompromised mouse strains (see later). As an alternative, *C. muris* has previously been employed for immunological studies (particularly by this group) but important arguments made against the use of this parasite are that it infects only the gastric glands and appears to be non-pathogenic (McDonald *et al.,* 1992).

In view of the difficulty of establishing *C. parvum* infection in adult mice, researchers have frequently turned to the neonatal infection model. Neonatal mice of most commonly used immunocompetent mouse strains, like newborn cattle or sheep, are highly susceptible to infection, but usually recover (Harp *et al.,* 1990). Surprisingly, few studies have examined the role of adaptive immunity against *C. parvum* infection of neonatal hosts. In one of the first mechanistic immunological studies of *Cryptosporidium*, Heine *et al.* (1984) observed that BALB/c neonatal wild type and athymic nude mice both initially recovered from *C. parvum* infection, but recovery was slower in the T cell-deficient mice and these animals rapidly had a relapse and died. This suggested, therefore, that T cells were important not just for elimination of the parasite but for the initial recovery during the early acute infection observed in neonates. Later studies, however, indirectly called into question a protective role for T cells during the acute phase of infection. One study failed to find *C. parvum* activated T cells in the spleen throughout the acute infection (Harp *et al.,* 1996). Another

investigation showed that $\beta7^{-/-}$ mice lacking the integrin α4β7 required for homing of mucosally activated T and B cells to the gut were not impaired in controlling the acute infection (Mancassola *et al.*, 2004).

Results from a recent study in this laboratory from Korbel *et al*. (2011) indicated further that T cells were not vital for the control of *C. parvum* infection in the neonatal mouse. Contrary to the findings of Heine *et al.* (1984) with their BALB/c nude mice described above, no difference in the pattern of acute infection was found between C57BL/6 wild type and $Rag2^{-/-}$ mice lacking T and B cells. For several weeks following recovery no oocysts could be detected in either mouse strain but treatment at that point with the immunosuppressive drug dexamethasone resulted in patent infection developing in the $Rag2^{-/-}$ mice but not wild type animals. Treatment of wild type neonatal C57BL/6 micewith anti-CD4 neutralizing monoclonal antibodies virtually eliminated $CD4^+$ T cells in the mesenteric lymph nodes and gut but, importantly, the mice did not have increased susceptibility to *C. parvum* infection. Also, the percentages of $CD4^+$ T cells and $CD8^+$ T cells in the lamina propria at the peak of the acute infection and during recovery did not increase. These findings strongly suggested that although adaptive immunity is required to eliminate *C. parvum* it is not important for control of the initial acute phase of infection of neonatal mice. So, $CD4^+$ T cells may be essential for control of infection in adult mice (see later) but are not involved in overcoming parasite reproduction in the neonatal mice. It remains to be determined whether the key role for innate immunity in mice described by Korbel *et al*. also applies to larger hosts such as human infants and newborn cattle. Also, the findings pose a question about the value of vaccination for the neonatal host.

1.2.3 Adaptive immune responses

1.2.3.1 Cell-Mediated Immunity

Normally, innate immune responses limit microbial replication early during infections but clearance requires a T cell-mediated immune response. The emergence of cryptosporidiosis as a common opportunistic infection in immunocompromised hosts, especially HIV patients, suggests that a cell-mediated mechanism is essential in the immunity against *Cryptosporidium*.

T lymphocytes are responsible for antigen-specific cell mediated immune responses. They consist of cytotoxic T cells and T helper (T_H) cells. Cytotoxic T cells usually express the $CD8^+$ molecule and recognise antigens in conjunction with MHC Class I molecules. T helper cells express the $CD4^+$ molecule and recognise antigen presented to them by antigen presenting cells which express MHC Class II molecules. Depending on the nature of the antigens that the immune system faces, $CD4^+$ T cells may trigger a cell-mediated immune response (T_H1) or antibody-mediated response (T_H2) (Mosmann and Coffman,1989).

These responses are differentiated depending on the cytokine spectrum produced by T cells and by antigen presenting cells. Thus T_H1 is recognised by the production of IFN-γ, IL-12 and TNF-α. A T_H2 response is associated with IL-4, 5,9,10 and 13 secretion. Intracellular pathogens induce T_H1 responses, while extracellular pathogens promoteT_H2 responses. T_H1 and $T_H2$responses can cross-regulate each other, and the outcome of an infection can depend upon the timing and magnitude of each response (Mosmann and Coffman, 1989). T_H17 cells are induced by IL-23 and they produce IL-17, IL-6 and TNF-α (Bettelli *et al.,* 2006). This response is commonly recognised in inflammation associated with autoimmune conditions (Weaver *et al.,* 2012).

1.2.3.1.1 The Role of T Cells

Infection with *C. parvum* causes a significant increase in the size of the T cell population in different mucosal compartments including Peyer's Patches, lamina propria and intraepithelial lymphocytes (McDonald, 2007). This is associated with an increase in expression of proinflammatory cytokines by T cells (White *et al.,* 2000). Infection also leads to villous atrophy and crypt hyperplasia (Alcantara Warren and Guerrant, 2007) that are characteristic of T cell induced intestinal pathology (MacDonald and Spencer, 1992).

Studies of murine infection models with *Cryptosporidium* confirm the importance of T cells in the immunity to this parasite. The first study that indicated the T cell role was made by Heine *et al*,who observed that neonatal nude mice developed a chronic and sometimes fatal *C. parvum* infection, while age-matched wild type mice had an acute self-limiting infection (Heine *et al.,* 1984). A similar observation was found in infection of adult nude and SCID mice with *C .parvum* (Ungar *et al.,* 1990; Mead *et al.*, 1991), although the adult mice initially showed strong resistance. The chronic infection in those animals was eliminated by injection of the mice with histocompatible T cells (Mead *et al*., 1991).

The role of T cells receptor types, TCRαβ and TCRγδ in resistance to *C. parvum* was investigated using transgenic mice lacking one of the TCR types ($TCR\alpha^{-/-}$ and $TCR\delta^{-/-}$ mice) (Waters and Harp, 1996). Whereas both neonatal and adult wild-type mice were able to control the infection, $TCR\alpha^{-/-}$ mice of both age groups suffered chronic infection In the case of $TCR\delta^{-/-}$mice, adult animals were as resistant to the infection as wild-type mice, but had delayed recovery when mice were infected as neonates (Eichelberger *et al.,* 2000). These observations therefore suggest that TCRαβ is essential for the control of infection, while TCRγδ, although not essential, takes a protective role in neonatal

infection. This may be in contradiction with work from this laboratory showing that neonatal Rag2$^{-/-}$ mice were no more susceptible to infection than wild type mice (Korbel *et al.,* 2011).

1.2.3.1.1.1 Role of CD4^{+} and CD8^{+} T cells

The correlation between CD4^{+} cell count and chronicity and severity of *C. parvum* infection in HIV patients was described by Blanshard *et al.* HIV infects mainly CD4^{+} T cells and causes a large depletion of these cells leading eventually to AIDS. The lower the CD4^{+} cell number the more severe and prolonged was cryptosporidiosis and mortality due to the parasite infection became more likely (Blanshard *et al*., 1992). Restoration of CD4^{+} T cells following antiretroviral therapy in HIV patients confers resistance to cryptosporidial infection (Farthing *et al.,* 2000). These observations support an important role for CD4^{+} T cells in immunity *C. parvum.*

Murine studies also supported the importance of CD4^{+} cells in the control of infection. While wild type mice were able to clear *C. parvum* infection after 8 weeks, MHC class II deficient mice that lack CD4^{+} cells remained infected by that time (Aguirre *et al.,*1994). Similarly immunocompetent mice suffered an exacerbation of infection following the administration of anti-CD4 antibodies (Ungar *et al.,* 1991). In studies with *C. muris* infection of adult SCID mice, immunity could be adoptively transferred to these animals with lymphocytes from spleens or mesenteric lymph nodes of immunocomeptent mice recovered from infection. This protective effect was lost when the donor cells from the immunocomeptent mice were depleted of CD4^{+} cells (McDonald *et al*. 1994). In line with this, a recent work by Tessema *et al.* indicated that adoptive transfer of CD4^{+} T cells and intraepithelial lymphocytes from IFN-γ$^{-/-}$ and IL-12p40$^{-/-}$ C57BL/6 mice infected with *C. parvum* to naive mice conferred protection against infection in the recipients (Tessema *et al.,* 2009b).

While $CD4^+$ T cells have been shown to be required for the host to control *Cryptosporidium* infection, the protective role for $CD8^+$ T cells has not been shown to be as important. Similar levels of infection were observed in mice that possessed or lacked $CD8^+$ T cells due to MHC class I deficiency (Aguirre *et al.,* 1994). Also, depletion of $CD8^+$ T cells from immunocompetent mice did not affect parasite reproduction. However, in the same study, there was a higher number of oocysts shed from mice depleted of both $CD4^+$ and $CD8^+$ T cells than from mice depleted of $CD4^+$ T cells only (Ungar *et al.,* 1990). In an investigation with *C. muris,* a role for $CD8^+$ T cells was observed astreatment with anti-CD8 antibodies enhanced parasite reproduction in mice, but was less effective than the treatment with anti-CD4 antibodies (McDonald *et al.,* 1994). Furthermore, Abrahamsen *et al.* suggested that $CD8^+$ cells could have an important role in resistance to *C. parvum* since histologically there was a significant increase in the number of these cells in the intestine of newly infected as well as reinfected bovine calves (Abrahamsen *et al.,*1997). In human studies, Pantenburg *et al.,* showed that antigen expanded sensitized $CD8^+$ T cells significantly reduced the quantity of *C. parvum* in human intestinal cell cultures in an HLA class I- dependent manner (Pantenburg *et al.,* 2010). Those effects were most likely mediated by the release of cytotoxic granules (Pantenburg *et al.,* 2010). Also, cryptosporidiosis infection was more common in humans with particular HLA-class I alleles (Kirkpatrick *et al.,* 2008).There have been no reports showing the presence of antigen-specific cytotoxic T cells *in vivo* in human.

Thus, an apparent discrepancy in the role of $CD8^+$T cells in different *Cryptosporidium* infection models and it is possible, therefore, that $CD8^+$ T cells may be more relevant for host resistance in human than in mice. Further studies of human mucosal $CD8^+$ T cells from patients are essential to establish the significance of these cells.

1.2.3.1.1.2 T_H1 and T_H2 responses

1.2.3.1.1.2.1 T_H1 response

Numerous studies have identified IFN-γ to be a major cytokine in the adaptive immune response to *Cryptosporidium*. A study by Ungar *et al,* described the important relationship between $CD4^+$ T cells and IFN-γ in *C. parvum* infection as mice which were treated with two neutralizing antibodies, one to IFN-γ and one to $CD4^+$ cells, developed an increase in level of infection in comparison to mice treated with either antibody alone (Ungar *et al.,*1991).

Treatment of immunocompetent mice with anti-IFN-γ neutralizing antibodies resulted in a significant increase in both *C. parvum* and *C. muris* reproduction but repeated antibody doses did not prevent eventual control of infection (Ungar *et al*., 1991 and McDonald *et al.,* 1992). Thus IFN-γ may be only required during the early part of infection. Furthermore, the reproduction of *C. parvum* in IFN-γ gene knockout mice was significantly greater than in the wild type mice, although the degree of increased susceptibility to infection of the knockout animals varied with the background strain: BALB/c mice survived but C57/BL mice died (Theodos *et al*, 1997; Mead and You, 1998). Also, high levels of IFN-γ expression were detected in intestines of infected mice, measured by RT-PCR or ELISA (Urban *et al.,* 1996; Kapel *et al.,* 1996).

Studies in humans also identified the role of IFN-γ in adaptive immunity as well as in treatment for cryptosporidiosis. Recovery of 15 patients from cryptosporidiosis was associated with IFN-γ release by peripheral blood mononuclear cells (PBMC) cultured with the parasite antigen. In a case report a two year old child with no immunodeficiency developed severe cryptosporidiosis and there was no IFN-γ from antigen stimulated PBMC (Gomez Morales *et al*., 1996).This cytokine was also used with success in the treatment of one child with chronic cryptosporidiosis (Gooi, 1994)

Although murine studies failed to show a protective effect for exogenous IFN-γ (McDonald and Bancroft, 1994; Kuhls *et al.,* 1994) in immunocompromised rats the treatment with the cytokine decreased the level of infection (Rehg, 1996). These findings supported the important role of IFN-γ in the early control of infection, but other mechanisms are also required in the absence of this cytokine.

The ability of TNF-α, another important T_H1 cytokine, to control the infection was examined in mice that lacked this cytokine. The key findings with neonatal TNF-$\alpha^{-/-}$ mice were that although TNF-α is expressed during infection and could directly inhibit parasite development in enterocytes *in vitro*, this cytokine was unnecessary for normal elimination of the parasite (Lean *et al.,* 2006).

In the T_H1 response IL-12 produced by dendritic cells and macrophages stimulates T cells to produce IFN-γ. A number of studies have shown the importance of IL-12 in the adaptive immune response to *Cryptosporidium*. The earliest evidence was provided by Urban *et al*. Treatment of immunocompetent neonatal BALB/c mice with IL-12 before inoculation with oocysts prevented or greatly reduced the severity of infection and resulted in increased IFN-γ expression in the intestine. On the other hand, the severity of *C. parvum* infection was exacerbated by treatment with anti-IL-12 antibodies and the protective effect of IL-12 was completely blocked by anti-IFN-γ antibodies (Urban *et al*.1996). IL-12 mRNA expression was also found to relate to the early control of infection of neonatal BALB/c mice (McDonald *et al.,* 2004). IL-12 deficient C57BL/6 mice were more susceptible to infection than wild-type mice. However, these mice were found to be able to produce IFN-γ in response to infection and could control infection, indicating the role of other cytokines for the induction of IFN-γ (Ehigiator *et al.,* 2007; Tessema *et al.,* 2009a).

The study by Ehigiator *et al.* also compared the infection in IL-12p40$^{-/-}$ mice (lack both IL-12 and IL-23) and IL-12p35$^{-/-}$ (lack IL-12) and suggested that IL-23 has no additional

role in *C. parvum* control as IL-12p40$^{-/-}$ were no more susceptible to infection than p35 knockout mice (Ehigiator *et al.,* 2007). As IL-23 is associated with T_H17, this result suggests T_H17 may not be involved in immunity to *C. parvum.*

Unlike the infection in neonatal BALB/c and C57BL/6 mice, adult BALB/c mice showed minor dependence on IL-12 as IL-12p40$^{-/-}$ mice developed mild infection similar to the wild type mice (Campbell *et al.,* 2002).

IL-18, similar to IL-12 promotes T_H1 responses (Cai *et al.,* 2000), it acts synergistically with IL-12 or other cytokines and is an important inducer of IFN-γ production by NK cell and T cells. Studies also suggest the involvement of IL-18 in the enhancement of T_H2 responses (Nakanishi*et al.*, 2001) . Studies have strongly supported the involvement of this cytokine during *C. parvum* infection. IL-18 is upregulated *in vivo* in response to infection (Ehigiator *et al.,* 2005; Tessema *et al.,* 2009a). Ehigiator *et al.* also observed that treatment of IL-12p40$^{-/-}$ knockout mice with exogenous IL-18 increased their resistance to infection, in addition IL-18$^{-/-}$ adult mice were more susceptible to infection (Ehigiator *et al.,* 2007). Furthermore, the treatment of IFN-γ knockout mice or IL-12-deficient mice with anti-IL-18 antibodies increased parasite excretion. This suggests that the protective role of IL-18 is not totally dependent on IFN-γ expression (Tessema *et al.,* 2009a). The same study also indicated that in IFN-$\gamma^{-/-}$ and IL-12$^{-/-}$ mice there was an increase in IL-4 and IL-13 expression in spleens of infected mice when treated with anti-IL18 antibody (Tessema *et al.,*2009a), supporting the role of IL-18 in polarization of T_H1 response against the parasite.

In summary, evidence strongly supports the role of T_H1 cytokines in the immunity against *C. parvum* and IFN-γ is a key player during the adaptive response.

1.2.3.1.1.2.2 T_H2 response

While the T_H1 pathway is vital in the immune response to *C. parvum*, particularly through the production of IFN-γ and IL-12, the effect of these cytokines was reported to decrease in the later stage of infection (McDonald, 2000). In fact, it has been suggested that a T_H2 response becomes involved in control of the later stage of the infection (Tessema *et al.,* 2009). However, the role of the T_H2 response in *C. parvum* infection is not that clear (Mcdonald, 2011).

Enriquez and Sterling studied the importance of a T_H2 response in infection and showed that treatment of adult mice with a combination of anti-IL-4 and anti-IL-5 antibodies increased the level of infection in comparison to the control animals. When given separately, anti-IL-5 had a more prominent effect than anti-IL-4, suggesting a more pronounced effect for IL-5 (Enriquez and Sterling, 1993). Another study also showed that IL-5 was expressed during infection of BALB/c IFN-$\gamma^{-/-}$ mice that recover from infection (Smith *et al.,* 2000). The evidence for a possible protective role of IL-4 is contradictory. In a study employing adult mice of C57BL/6 background, no increase in vulnerability to infection was observed in adult C57BL/6 IL-$4^{-/-}$ mice in comparison to control animals (Campbell *et al.,* 2002) and in agreement with this, treatment of adult C57BL/6 IFN-$\gamma^{-/-}$ mice with anti-IL-4 antibodies did not result in a significant difference in the level of infection when compared to control animals (Petry *et al.,* 2010). Other studies, however, indicated a significant role for IL-4. Aguirre *et al.,*demonstrated that in adult C57BL/6 mice resolution of infection was associated with increased numbers of IL-4 producing $CD4^+$T cells in the gut-associated lymphoid tissue and furthermore treatment with anti-IL-4 antibody caused a prolongation of patent infection (Aguirre *et al.,* 1998). In agreement with this, neonatal BALB/c mice treated with anti-IL-4 antibody and BALB/c IL-$4^{-/-}$ mice had higher susceptibility to infection than the control mice

(McDonald *et al.,* 2004). A recent study by Tessema *et al.* showed that in IFN-$\gamma^{-/-}$ mice significant expression of IL-4, IL-10 and IL-13 was detected in the intestine and spleen and the levels mirrored the pattern of oocyst shedding. Those cytokines were also measured in the intestine of IL-$12^{-/-}$ mice, but the expression did not match the infection level, but continued to rise when oocyst shedding was decreasing. All cytokines fell to basal levels at the resolution of infection. Both IL-4 and IL-13 were significantly increased in both knockout mouse strains after treatment with anti-IL-18 antibody that worsened infection. No change in IL-5 expression was observed (Tessema *et al.,* 2009). It was therefore suggested that in the absence of T_H1 cytokines, the immune response shifts to a T_H2 type response that is much less efficient but eventually establishes control. It is possible, therefore, that normally balanced T_H1 and T_H2 responses are involved in the resolution of infection (Tessema *et al.,* 2009).

In summary, there is strong evidence for a major role for T_H1 cytokines, particularly IFN-γ in the control of *Cryptosporidium* infection. However, T_H2 cytokines may also contribute to effective control of infection especially in the latter stage of the infection. At present the involvement of T_H17 in *C. parvum* infection is unclear and needs to be investigated.

1.2.4 Humoral Immune Responses

1.2.4.1 B cells and antibody responses

In *C. parvum* infection, antibodies of all major classes are produced and high titres were measured in the mucosa and circulation of different hosts, including humans, cattle and sheep (Ungar *et al.,* 1986; Peeters *et al.,* 1992; Hill *et al.,* 1990). IgM, IgG and IgA titres measured by ELISA generally increase during infection and decline after recovery (Ungar *et al.,* 1986). In a study with adult human volunteers, however, secretory IgA was detected in fecal samples during infection but neither IgG nor IgM was found (Dann *et al.,* 2000). In developing countries IgG titres in serum of children may continue to elevate with time, probably because of continuous exposure to the parasite (Priest *et al.,*2006) but it was reported that IgA and IgM levels declined in cases with persistent diarrhoea (Khan *et al*., 2004). A recent study with IFN-$\gamma^{-/-}$ mice or animals with IL-12 deficiency demonstrated that infected animals mounted prolonged parasite-specific serum IgG and IgA responses (Jakobi and Petry, 2008). Moreover, challenge infection led to a booster effect in immunoglobulin response despite the apparent absence of oocyst shedding (Jakobi and Petry, 2008).

The protective role of antibodies in *C. parvum* infection is still questionable. Patients with congenital hypogammaglobuilinemia were unable to clear *C. parvum* infection (Lasser *et al.,*1979), implying that antibodies are required for immunity. This was supported by a study with AIDS patients with a strong serological response to a 27-kDa parasite antigen associated with a reduced risk of cryptosporidial dairrhoea (Frost *et al.,* 2005). In contrast, other studies of severe *Cryptosporidium* infection in AIDS patients with low CD^+ T cell counts showed high titres of IgA and IgG in their serum as well as salivary IgA (Cozon *et al.,* 1994; Kaushik *et al.,* 2009).

Mice were partially protected against *C. parvum* infection after injection with gall bladder secretory IgA (sIgA) from rats recovered from infection (Albert *et al.,* 1994). Similarly, monoclonal sIgA that recognised *C. parvum* surface antigen p23 prepared from Peyer's patch B cells from infected mice was able to provide a degree of protection to neonatal mice after passive transfer (Enriquez and Riggs, 1998). This suggest that sIgA plays a part in immunity to this parasite in mice but other investigations with B cell-deficient neonatal mice or when B cells were depleted by treatment with anti-μ chain antibodies indicated a limited role as those mice recovered from infection as well as controls (Chen *et al.,* 2003; Taghi-Kilani *et al.,* 1990).

Thus, although antibodies may contribute to protective immunity against *Cryptosporidium*, evidence indicates that antibodies alone do not clear infection and other mechanisms are needed for infection control.

1.2.4.2 Passive transfer of anti- *C. parvum* antibodies

Different studies have investigated the protection against *Cryptosporidium* with antibodies obtained by various methods of immunization and the results were inconsistent. Variable results were obtained from the immunisation with hyperimmune colostrums (HBC) which was prepared after injection of crude *C. parvum* oocyst antigen into the mammary glands of preparturient cattle (Tzipori *et al.,*1986; Fayer *et al.,* 1989a). Oral administration of HBC to humans with severe disease was, in some instances, shown to decrease the parasite burden and symptoms (Tzipori *et al.,*1986; Ungar *et al.,* 1990). However, in another study of healthy volunteers, no significant prophylactic effect for HBC was observed (Okhuysen *et al.,* 1998). Treatment of calves with HBC decreased the duration of diarrhea and oocyst excretion (Fayer *et al.,* 1989a). In murine infection, a lower level of infection was observed in HBC-treated neonatal mice in comparison to the control animals (Fayer *et al.,* 1989b). Antibodies isolated from HBC attached sporozoites when incubated together and reduced their infectivity when transferred to neonatal mice (Fayer *et al.,* 1989b).

Colostrum from a cow immunised with CP 15/60 based DNA vaccine transferred to drug-immunosuppressed mice induced partial protection against *C. parvum* infection (Jenkins *et al.,* 1999). Also, in this regard, good protection against *C. parvum* was achieved in calves receiving colostrum from cows vaccinated subcutaneously with recombinant p23 antigen that is present in both sporozoites and merozoites, plus adjuvant (Perryman *et al.,* 1999).

C. parvum immunized chickens produced eggs containing high titres of anti-*C. parvum* antibodies that reduced binding of sporozoites to enterocytes *in vitro* and when transferred orally to SCID mice supplied partial protection against infection (Cava and Sterling,1991; Kobayashi *et al.,*2004).

Evidence of protection with monoclonal antibodies (mAb) was also described. Incubation of sporozoites with certain mAb that recognise surface antigens reduced their infectivity for neonatal mice in a time-dependent manner (Perryman *et al.,* 1990). mAbs to individual antigens given orally to neonatal mice before infection did not provide any immunity, but when mAbs to different antigens were used in combination on three consecutive days from the time of infection the infection level was decreased (Arrowood *et al.,* 1989). Similarly, protection against *C. parvum* infection was achieved by treatment of neonatal mice with a mixture containing three mAbs to surface antigens but individual mAbs were not protective (Schaefer *et al.,* 2000). In contrast, Riggs *et al* demonstrated that treatment of adult SCID mice with a single mAb (3E2) reduced parasite reproduction by binding with a sporozoite surface antigen: (circumsporozoite-like glycoprotein (CSL)). However, when used in combination with other antibodies there was no increase in efficacy (Riggs *et al.,* 2002).

1.2.4.3 Vaccination against *Cryptosporidium* infection.

Cryptosporidiosis is a major health risk in developing countries and, as there is no reliable chemotherapeutic agent it would therefore be ideal to develop a vaccine against the disease. However, little direct work has been undertaken in that field and this is probably due to numerous scientific obstacles that need be overcome before vaccines could be available.

Attenuated *Cryptosporidium* using γ-irradiation of oocysts was shown to reduce parasite reproduction after oral administration and induced partial resistance against subsequent challenges with viable oocysts (Jenkins *et al.,* 2004).

As reviewed by Boulter-Bitzer *et al.,* many immunogenic antigens of the *C. parvum* invasive stages involved in attachment or penetration of host cells have been identified (Boulter-Bitzer *et al.,* 2007) and immunisation of mice with antigen DNA has produced high levels of antibody, IFN-γ producing T-cells and some protection against infection (Ehigiator *et al.,* 2007; Zheng *et al.,* 2011). Therefore, some antigens might be useful for vaccines, however, it is not known if the parasite antigens that have been recognised are the same ones that provide protective immunity (McDonald, 2011).

Mucosal administration of non-living vaccines often fails to induce an effective immune response; this could be due to oral tolerance that prevents inflammatory response against harmless antigens, inadequate stimulation of pathogen-recognition receptors and/or failure to enter the M cell pathway for immune recognition (Pasetti *et al.,* 2011). The use of parenteral immunisation to stimulate mucosal protection also usually fails, most probably due to the lack of expression of intestinal homing molecules by activated T cells (Pasetti *et al.,* 2011).

In regard to vaccination of newborn livestock, due to the development of natural resistance to infection, protection is only required for a relatively short period early in life and the immature

immune system of neonates may not respond well to vaccination (McDonald, 2007). Moreover, as described by Korbel *et al.,* in newborn mice,adaptive immunity appears to have a significant role in controlling the infection than innate immunity (Korbel *et al.,* 2011). Therefore, it might be more useful to target older livestock with vaccination in order to reduce the general level of oocyst contamination in the local environment (McDonald, 2011).

In humans, vaccination might have real relevance in areas of developing countries with poor hygiene and hence a high prevalence of infection and it might be important to immunise against more than one species of *Cryptosporidium* (McDonald, 2007).

In summary, further studies and approaches to establish effective and cost benefit vaccines against infection are required.

1.3 GENERAL HYPOTHESIS AND AIMS

It is clear from many studies that adaptive immunity is essential for the elimination of cryptosporidial infection. It is also evident that although innate immunity alone cannot clear infection it can play an important part in the control of parasite reproduction. Significantly, studies from this lab suggest that recovery during acute *C. parvum* infection in neonatal mice is due to innate immunity. The components of innate immunity that are involved in protection against *C. parvum* are not well characterised, however.

Both type I IFN and NK cells are known to be important in innate immunity against many viruses and certain bacteria, but their roles in resistance to infection by *Cryptosporidium* have not been clear.

MAIN HYPOTHESIS:

Type I IFN and NK cells are vital elements of the protective innate immune response against *C. parvum.*

AIMS

1. Use in vitro culture systems to investigate the potential role of type I IFN and NK cells in immunity.
2. Employ murine infection models to confirm that type I IFN and NK cells are important factors in the innate immune response that inhibits parasite reproduction.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 Materials

A variety of plastic consumables were used for tissue culture and other techniques. Centrifuge tubes, tissue culture flasks, multi-well tissue culture plates (6, 24 and 96 wells), petri-dishes, pipettes, plastic pastettes and ELISA plates were obtained from VWR International. Frosted glass microscope slides and slide coverslips were also obtained from VWR International.

2.2 Parasite preparation

C. parvum oocysts of the IDAHO isolate were obtained from Bunch Grass Farm, Deary, Idaho, USA and stored at 4°C in PBS. Oocysts were re-suspended in a 10% commercial bleach solution (0.55% sodium hypochlorite) for surface sterilization. The parasites were then washed in PBS (pH 7.2) 3 times by centrifugation at 3,000xg for 6min in a microfuge. The oocysts were then re-suspended in PBS and counted microscopically using a haemocytometer (McDonald *et al*., 1992).

2.3 Cell Culture

The human adenocarcinoma cell line Caco-2, the murine rectal adenocarcinoma cell line CMT-93 and the L929 murine fibroblast cell line were used. Cell monolayers were maintained in Dulbecco Modified Eagle Medium (DMEM) with 10% fetal bovine serum, 100µgm/ml streptomycin, 100U/ml penicillin, 4 mM L- glutamine and 1% non essential amino acids (complete medium, all from Invitrogen Life Technologies). Cells were grown in large or small plastic flasks at 37°C in an incubator with 5% CO_2 and 95% air. Cell suspensions were obtained for passage or experiments by treatment with 0.25% trypsin (Sigma–Aldrich). Cells were washed in complete DMEM by centrifugation at 500xg, resuspended in complete medium and then added to flasks or multi-well plates (Pollok *et al.,* 2001).

In some experiments Caco-2 or CMT-93 cells were seeded on sterile 13mm diameter glass cover slips placed on the bottom of 24-well plates to allow for later removal and attachment to glass slides for microscopic examination of monolayers at high magnification.

2.4 *C. parvum* infection of cells

When monolayers were around 80% confluent, the medium was discarded and cells were infected by addition of 2×10^5 *C. parvum* oocysts in 250µl complete medium for 24-well plates or 1ml of medium for 6-well plates. After 120-180 min incubation at 37°C which is the time needed for sporozoite excystation and cell invasion, cell monolayers were washed twice with fresh medium to remove oocyst debris plus any unattached parasites and cells were further cultured for 24 h in normal volumes of fresh medium (Lean *et al.,* 2006).

In some experiments, CMT-93 monolayers were infected with purified sporozoites.

2.5 Mice

Neonatal and adult BALB/c, BALB/c SCID mice, C57BL/6 Rag-$2^{-/-}$ and C57BL/6 Rag-$2^{-/-}$ γc$^{-/-}$ mice were bred and maintained in filtered cages under specific pathogen-free conditions. All procedures performed were agreed with a local ethical committee and licensed by the United Kingdom Home Office.

2.6 Animals' infection

Mice were infected by oral gavage either as neonates (usually 7days of age) normally using 1×10^4 oocysts or, as weaned animals (4-8 weeks of age) using 1×10^4-1×10^6 oocysts depending on the nature of experiment (McDonald and Bancroft, 1994).

2.7 Measurement of infection

2.7.1 *In vitro* quantification of infection

This staining method was described by Bary and Garnham (1962). After 24h of infection, cell monolayers on coverslips were washed with PBS pH7.2, fixed with methanol and stained with 10% Giemsa in PBS for 2 h at room temperature. The coverslips were then washed in deionised water and glued to glass slides with Depex (BDH). Four monolayers were used per treatment. The parasite numbers (usually trophozoites/developing meronts) were then counted microscopically in 20 random fields under X1000 magnification with oil immersion.

2.7.2 Measurement of oocyst production in faecal material

The level of infection in mice was usually assessed by microscopic counting of oocysts in faeces. Stools or colonic contents were collected on different days postinfection and smeared evenly onto microscope slides. The smears fixed with methanol and acid-fast stained by the Zeihl Neelsen method. Slides were immersed in carbolfuchsin (BDH) for 30 min, de-stained with 1.5% vol:vol acid-alcohol (conc. HCl and methanol), counterstained with 0.5% w/vol malachite in distilled water and washed with tap water. The numbers of parasites were then counted microscopically in 50 random fields at X1000 magnification using oil immersion (Baxby *et al.,* 1984). (Fig 2 shows slides with acid-fast stained faecal smears). With this method oocysts have a pink colour that is easily detected on a blue-green background (see later).

Figure 2. Stool samples smears stained by the Zeihl Neelsen method

2.7.3 Measurement of *C. parvum* infection in intestinal sections from neonatal SCID mice

Early stages of infection in mice were measured semi-quantitatively by counting parasites developing in intestinal villi. One day after treatment with anti-Type I IFN antiserum, seven day-old mice were infected by inoculation with a much larger than normal number of oocysts ($1X10^5$) so that parasites can be seen early during infection. At 48h postinfection the ileum was removed and placed in formal saline solution. The tissue was embedded in paraffin and 5μm longitudinal sections prepared and stained with haematoxylin and eosin. Ten villi were selected at random and given an infection score of 0-10 (0, no parasites; 1, up to 10% of cells infected; etc. up to 10 for all cells infected). A total score (highest possible being 100) was obtained for each mouse and the mean calculated for the group.

2.8 RNA extraction

Total RNA from intestinal tissue samples, the epithelial cell line CMT-93 or bone marrow derived dendritic cells was isolated using a monophasic solution of phenol and guanidine thiocyanate (TRIZOL, Invitrogen) according to the manufacturer's instructions, followed by chloroform extraction and iso-propanol precipitation. Total

RNA was quantified by spectrophotometry and 3µg RNA was reverse transcribed to cDNA at 42°C with 1.5µg oligo(dT) primer (Promega), 1mM deoxynucleoside triphosphate, and Moloney murine leukaemia virus reverse transcriptase in a volume of 20µl, following the manufacturer's guidelines. cDNA were either kept at -20°C or used for real time qPCR.

2.9 Real-time quantitative PCR

Reaction mixtures were set up to a final volume of 20µl using a total of 100ng cDNA, 20 pmol of each primer and 10µl FastStart SYBER Green master mix (Roche). Amplification was performed using a Rotor-Gene 3000 instrument (Corbett Research) three times with independent cDNA samples and in triplicate for each cDNA and primer pair (The technique used followed the manufacture instructions).

The PCR protocol consisted of an initial hold step of 95°C for 10 min followed by 45 cycles of amplification under the following conditions: denaturation at 95°C for 15 sec, annealing at 60°C for 30sec and elongation at 72°C for 60sec.

The comparative threshold method was used for relative quantification ($\Delta\Delta C_T$ method). The amount of target gene was normalised to the housekeeping gene β-actin, and relative to the calibrator. Amplification efficiencies E for the housekeeping gene and all target gene reactions were determined ($E_{\beta\text{-actin}}$ = 0.932; $E_{IFN\text{-}\alpha}$= 0.902; $E_{IFN\text{-}\beta}$ = 0.965; $E_{IFN\text{-}\gamma}$ = 0.919) by preparing a dilution series with cDNA template amounts between 0.01ng and 100ng, plotting the C_T values obtained against the logarithm of the template amounts to construct a standard curve, and calculating amplification efficiencies using the formula $E = 10^{(-1/S)} - 1$, where S is the slope of the standard curve.

2.10 Enzyme-linked immunosorbent assay (ELISA)

ELISA technique was used to measure the release of IFN-α, IFN-γ or IL-12 in supernatants of cultured cells. The procedures were performed following the manufacture guidelines for each cytokine. All ELISA kits were supplied by R&D system.

The level of IFN-γ or IL-12 production in supernatants of cultured splenocytes was measured by ELISA kits from R & D Systems and employing the protocols and reagents provided by the manufacturers. Precise details of concentrations of some reagents are not known. A 96-well ELISA plate was coated with 100µl of diluted capture antibody overnight at room temperature. The wells were then aspirated and washed twice with the washing buffer. The plate was dried by tapping it face down vigorously on a dry paper towel several times until no wet spots appeared on the towel. Following this there was an incubation for 1h with 300µl in each well of the specific blocking buffer. After washing and drying the wells, 100µl of samples or standards in reagent diluent were added and incubated for 2h. After washing, each well was coated with 100 µl of detection antibody and left for 2h. The wells were then emptied, washed and filled with 100µl of the working dilution of Streptavidin-HRP (horseradish peroxidase) and left in the dark for 20min. The plate was then washed and coated with 100µl of the substrate solution H_2O_2/tetramethylbenzidinefor 20min in the dark. The reaction was then stopped by adding 50µl of stop solution, 1N H_2SO_4. Using a microplate reader the optical density was measured at 450 and 570nm.

2.11 Antiviral bioassay

To quantify Type I IFN release in culture supernatants obtained from epithelial cells or dendritic cells, an established bioassay was used (Daffis *et al.,* 2007). 2×10^4 L929 murine fibroblast cells in complete medium were seeded in a 96-well plate and

cultured overnight at 37°C. The monolayers were then exposed to supernatants from treated and untreated cells (eg infected or uninfected epithelial cells) or to standard dilutions of recombinant mouse IFN-α_4 (R & D Systems) for 24h. The supernatants were then discarded and the L929 cells were exposed for 1h to encephalomyocarditis virus particles (ECMV in medium containing only 2% FCS, multiplicity of infection 0.5). Some control cells remained uninfected. The virus-containing medium was then removed and the cells were re-cultured in complete fresh medium for 16h. At the end of the incubation period the cells were fixed with 4% paraformadyhyde in PBS and stained with 0.25% crystal violet. Absorbance was then read at 580nm and the anti-viral activity of supernatants was measured in comparison to the recombinant IFN-α standards.

2.12 Preparation of splenocyte cultures

As described by McDonald *et al.* (1994), spleens from adult SCID, Rag $2^{-/-}$ or Rag $2^{-/-}\gamma c^{-/-}$ knockout mice were collected under sterile conditions and placed in ice cold RPMI-1640 (Invitrogen International Technologies) with 10% fetal bovine serum, 100µgm/ml streptomycin, 100U/ml penicillin, 4 mM L-glutamine, 1% nonessential amino acids and 5µM mercaptoethanol. The spleens were then disrupted in a sterile petri-dish containing complete medium using a 10ml syringe plunger and passed through 40µm sterile filters to get rid of any fatty or connective tissues. To lyse RBC, the cell suspension was then incubated for 10 min at room temperature with lysing buffer (0.83% w/vol ammonium chloride solution). The cells were then washed and resuspended in fresh RPMI. A total of 4×10^5 cells in 200 µl medium was seeded into each well in a 96 well plate and cultured for 24-48h with or without *C. parvum* oocyst antigen.

2.13 Flow cytometry studies (FACS)

5×10^5- 1×10^6 cells from spleens or bone marrow derived dendritic cells were washed twice in FACS buffer (PBS, 0.1% w/vol sodium azide solution (Sigma-Aldrich) and 1% fetal bovine serum) for 10 min at 500xg. The cells were resuspended in FACS buffer and fluorochrome-conjugated antibodies to cell surface markers were added. Each antibody dilution was determined by antibody titration. After incubation at 4°C for 30 min in the dark, the cells were washed in FACS buffer, fixed for 15 min at room temperature in fixationbuffer (PBS, 2% paraformaldehyde), washed and resuspended in FACS buffer (Cooper and Caligiari, 2003). The cells were then either analysed immediately or kept in the dark at 4°C for later analysis.

FACS antibodies: Antibodies used in flow cytometric analyses were: Rat anti-mouse CD49b (clone DX5) conjugated to fluorescein isothiocyanate (FITC; from BD Pharmingen) and mouse anti-mouse NK1.1 (clone PK136) conjugated to allophycocyanin (APC; from eBioscience). Isotype-matched control antibodies were: Rat IgM-FITC and mouse IgG_{2a}-APC (both from BD Pharmingen). Flow cytometric analyses were performed using a Becton Dickinson LSRII instrument and FACSDiva software.

2.14 Statistical analysis

Mean values ± standard error were calculated and statistical significance determined using Student's *t* test or ANOVA one way test.

CHAPTER THREE

THE ROLE OF TYPE I IFN IN THE INNATE IMMUNITY TO *CRYPTOSPORIDIUM PARVUM*

3.1 GENERAL INTRODUCTION

3.1.1 Interferons

Interferons (IFNs) are a family of structurally related cytokines with a general property of antiviral function. They are only found in vertebrates (Isaacs and Lindenmann, 1957). They exhibit a variety of biological functions represented by three major activities: antiviral activity, antitumor activity and immunomodulatory effects (Takaoka and Yanai, 2006). Interferons were the first cytokines to show efficacy in the treatment of viral infections and malignancies and the protocols have been used as models for the clinical development of other cytokines (Parmar and Paltanias, 2003).

3.1.2 Classification

Interferons are comprised of biochemically and functionally different proteins. There are three recognized classes of IFNs: type I, type II and type III according to their amino acid sequences.

Initially, IFNs were classified into classical or type I IFN and immune or type II IFN. This classification was based on their resistance or sensitivity to acid (pH2) and heat ($56^{o}C$) and their induction by viruses or immunostimulants (Ho and Armstrong, 1975). Type I IFNs were then subdivided depending on the virally infected target cell into leukocyte IFN and fibroblast IFN. The classification of leukocyte, fibroblast and immune IFN is no longer valid as leukocytes can produce all types of IFN. The classification of IFNs is now based on their amino acid sequence, chromosomal location and receptor specificity (Pestka *et al.,* 2004).

3.1.2.1 Type I IFNs

The type I IFNs consist of IFN α, β, ω, ε (Roberts *et al*., 1998; Langer *et al*., 2004; Pestka *et al*., 2004) and -κ (LaFleur *et al*., 2001). In addition, IFN-δ (Lefevre *et al*., 1998), -τ (Roberts *et al*., 1999) and –ζ (Oritani *et al*., 2000) are included in this group, although they are only detected in pigs/cattle, ruminants and mice, respectively. All the members of type I IFNs family transmit signals through a receptor complex composed of two subunits, IFNAR-1 and IFNAR-2 although there are some differences in both quality and efficiency in signalling among them (Takaoka and Yanai, 2006).

The human type I IFN family includes thirteen IFN-α genes (α1, α2 etc.), one IFN-β and two IFN-ω genes, all located on the short arm of chromosome 9 (Hardy *et al.,* 2004).The mouse type I IFN locus is on chromosome 4 and contains 14 IFN-α genes, a single IFN-β, ω and ε and a not yet defined number of IFN- ζ genes (Robert *et al.,* 1998; Hardy *et al.,* 2004).

3.1.2.2 Type II IFN

Type II IFN consists of a single IFN-γ gene that is located on chromosome 12 in humans and on chromosome 10 in mice (Bach *et al.,* 1997, Pestka, 1997). It signals through a receptor composed of IFNGR-1 and IFNGR-2 subunits (Bach, *et al.,* 1997).

IFN-γ plays a major role in the host's innate and T cell dependent responses to intracellular microorganisms. In response to mitogenic or antigenic stimuli, IFN-γ is produced by activated cells of the immune system, mainly T cells or NK cells (Bach *et al.,* 1997; Ikeda *et al.,* 2002). Some cytokines induce IFN-γ production such as IL-18, IL-12 and type I IFNs and they either function alone or synergistically (Dinarello, 1999).

3.1.2.3 Type III IFNs

These consist of three IFN-λ molecules called IFN-λ1, IFN-λ2 and IFN-λ3, also called IL-29, IL-28A and IL-28B, respectively (Kotenko *et al.,* 2003; Sheppard *et al.,* 2003). IFN-λ genes are located on chromosome 19. Type III IFN is induced upon viral infection and binds to a distinct membrane receptor complex of a specific chain IFNLR1 and a second chain IFNRL2/IL10R2 shared with IL-10, IL-22 and IL-26 (Kotenko *et al.,* 2003; Sheppard *et al.,* 2003).

3.1.3 Main IFN signalling pathways

3.1.3.1 IFN Receptors

Both type I and type II receptors are transmembrane glycoproteins whose extracellular domains act as IFN binding sites while the cytoplasmic domains associate with members of the JAK protein tyrosine kinases family (Jak PTKs) and initiate signal transmission (Prejean and Colamonici*,* 2000).

The IFNα's, -β and -ω's have a common receptor consisting of two subunits, IFNAR-1 and IFNAR-2. Both IFNAR-1 and IFNAR-2 map to chromosome 21 in the human, and chromosome 16 in the mouse. There is a single form of the IFNAR-1 subunit. However, alternative processing of the IFNAR-2 gene transcript produces long (2c), short (2b), and soluble (2a) forms of the encoded subunit (Mogensen *et al*.1999). IFNAR-1 associates with Tyk2 tyrosine kinase, whereas IFNAR-2 associates with Jak1 kinase (Platanias and Fish, 1999).

IFN-γ binds to a receptor distinct from that used by IFN-α/β. Two kinds of subunits also constitute the IFN-γ receptor complex. The IFN-γ ligand-binding IFNGR-1 subunit and the accessory IFNGR-2 subunit map to chromosomes 6 and 21 in the human and

chromosomes 10 and 16 in the mouse, respectively (Bach *et al.,* 1997). IFNGR-1 and IFNGR-2 are associated with the Jak1 and Jak2 kinase, respectively (Bach *et al.,* 1997).

3.1.3.2 IFN signalling pathways

3.1.3.2.1 Jak-Stat pathway

Classically, binding of type I IFNs to their receptors results in the activation of the Jak PTK (Tyk2 and Jak 1) that then phosphorylate their downstream substrates Stat1 and Stat 2 that are members of the family of signal transducers and activators of transcription (Stats). The tyrosine phosphorylation of Stats leads to the formation of two transcriptional activator complexes, IFN-α activated factor (AAF) also called IFN-γ activated factor (GAF) and IFN-stimulated gene factor 3(ISGF3). AAF/GAF is a homodimer of Stat1, while ISGF3 is a heterotrimeric complex of Stat1, Stat2 and IRF-9/p48/ ISGF3γ, another transcription factor member (Bluyssen *et al.,*1996). These complexes translocate to the nucleus and bind to specific DNA sequences namely IFN-γ activated site (GAS) and the IFN-stimulate regulatory element (ISRE) This leads to the activation of a large number of target genes (IFN-stimulated genes) to stimulate biological responses (Fig 3).

In addition to the classical Stat1 and Stat2 signalling, Stat3, Stat4 and Stat5 have been also found to be components of the type I IFNs signalling machinery (Fish *et al*. 1999)

3.1.3.2.2 Other signalling pathways of type I IFNs

Several studies indicate that type I IFNs can activate other signal cascades apart from the Jak-Stat pathway. Such pathways include cascades involving the CBL proto-oncogen, the CrkL adapter and the related CrkII protein (Ahmed *et al.,* 1997). This pathway is thought to mediate induction of the growth suppressive effects of IFNs.

Another important pathway is the insulin receptor substrate (IRS) signalling pathway. This is known to play a role in insulin and growth factor signalling. It has also been reported that the mitogen-activated protein (MAP) kinases, extracellular–signal–regulated kinase 2(ERK2) and p38 are activated by IFN-α/β (David *et al.,*1995; Goh *et al.,* 1999).

3.1.3.2.3 IFN-γ signaling

Unlike Type I IFNs, IFN-γ signalling occurs predominantly through the Stat1 pathway. Upon binding, the IFN-γ subunits dimerise leading to the activation of associated Jak1 and Jak 2 kinases. These then phosphorylate Stat1 which then forms a homodimer, translocates to the nucleus and activates transcription by binding to the GAS sequences (Platanias and Fish 1999; Darnell *et al.,* 1994). As mentioned previously, Type I IFN also signals using this pathway.

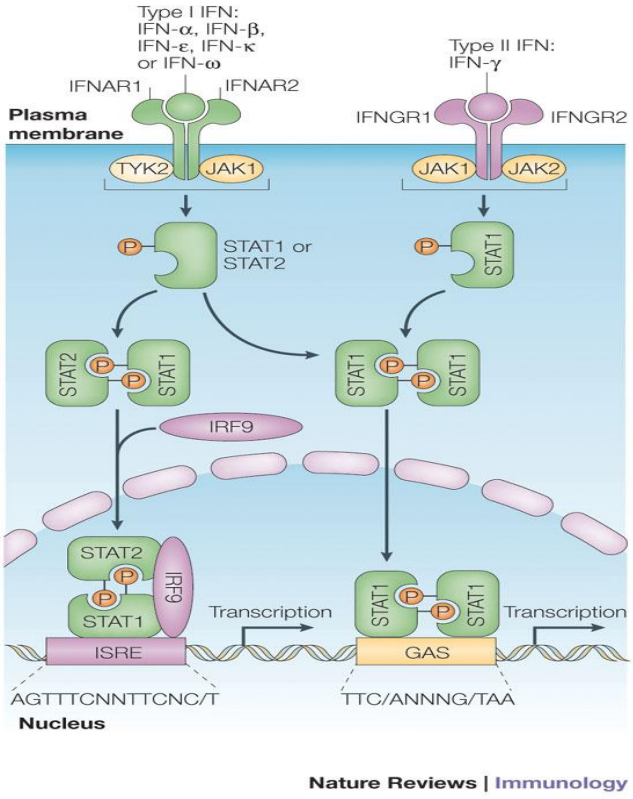

Figure 3. Principal type I and type II IFN signalling pathways

Platanias, Nature Reviews Immunology (2005).

All type I interferons (IFNs) bind a common receptor at the surface of the cells, which is known as the type I IFN receptor. The only type II IFN, IFN-γ, binds a distinct cell-surface receptor, which is known as the type II IFN receptor. Activation of the JAKs that are associated with the type I IFN receptor results in tyrosine phosphorylation of STAT2 and STAT1; this leads to the formation of STAT1–STAT2–IRF9 (IFN-regulatory factor 9) complexes, which are known as ISGF3 (IFN-stimulated gene (ISG) factor 3) complexes. These complexes translocate to the nucleus and bind IFN-stimulated response elements (ISREs) in DNA to initiate gene transcription. Both type I and type II IFNs also induce the formation of STAT1–STAT1 homodimers that translocate to the nucleus and bind GAS elements that are present in the promoter of certain ISGs, thereby initiating the transcription of these genes. The consensus GAS element and ISRE sequences are shown. N, any nucleotide.

3.1.4 Type I IFN production and induction

3.1.4.1 Type I IFN production

Although many cell types can express IFN-α or IFN-β in response to an appropriate viral, mitogenic or microbial stimulus, some cells are capable of producing larger amounts of IFN- α/β required for NK cell–mediated killing of virus-infected cells. These cells are called 'natural interferon producing cells' (NIPCs). They were first identified in human blood (Trinchieri *et al*., 1978; Ronnblom *et al*., 1983), and more recently in the mouse (Asselin-Paturel *et al*., 2001). They were identified as rare cells in human peripheral blood, exhibiting plasmacytoid morphology and lacking haematopoeitic markers (Fitzgerald-Bocarsly, 1993). The type I IFNs produced by these cells act systematically to induce an antiviral state and/ or stimulate other cells to express IFN-α/β (Sato *et al*., 2003). Natural IFN-producing cells are now referred to as plasmacytoid dendritic cells (pDCs) because of their round morphology, eccentric nucleus, and abundant endoplasmic reticulum (Colonna *et al*., 2004). pDCs preferentially express Toll-like receptor (TLR)7 and TLR9 (see later), allowing them to respond to single-stranded RNA and DNA viruses, respectively, by triggering signal transduction through the adaptor protein MyD88 (Colonna *et al*., 2004). These receptors are efficient in inducing type I IFN only in pDCs because these cells constitutively express IRF7 and IRF8, and the MyD88–IRF7 complex undergoes a spatiotemporal regulation upon TLR ligation such that it is retained in the endosomal compartment, where it induces type I IFN production (Colonna *et al*., 2004).

3.1.4.2 Induction of type I IFNs

Induction of type I IFN is governed by IFN regulatory factors IRF3, IRF7 and IRF5. The classical pathway of IFN induction was originally described in fibroblasts. Virus stimulation leads to thephosphorylation of IRF-3, its translocation to the nucleus and subsequent up-regulation of asubset of early type I IFN genes. These IFNs are translated, then secreted, and signal through the IFNAR and the JAK/STAT pathway to up-regulate IRF-7 expression which is needed for the transcription of the full range of the IFN-α genes and also for maximal expression (Honda *et al*.,2005)

The pDC induction of IFN is dependent on MyD88 and IRF-7 but not IRF-3. IRF-7 undergoes virus-induced phosphorylation, translocates to the nucleus and stimulates the production of multiple IFN-α subtypes after binding to IRF-binding elements (IRF-Es) I and III and PRD-like elements (PRD-LES) (Honda *et al.,* 2005)

In addition to viruses, polyriboinosinic:polyribocytidylic acid(poly (I:C)), certain cytokines (including IL-10), mitogens, tumor cells and a number of microbes and microbial products have been found to induce type I IFN production both in vivo and in vitro (Bogdan *et al.*, 2004).

3.1.4.3 Toll-like receptor-dependent and independent induction of type I IFNs

TLRs are a family of pathogen recognition receptors that play an important role in innate immunity against a range of microorganisms. Each TLR has its own signalling pathway and induces specific responses against different microorganisms. In humans, TLR3, 4, 7, 8 and 9 were all found to induce type I IFNs (Noppert *et al*., 2007).

Both TLR3 and TLR4 are able to induce IFN-β by activation of IRF-3 (Doyle *et al.,* 2002). Both TLR3 and TLR4 use the TRIF pathway via IRF-3 for type I IFN

induction,however there are some differences between the two receptors (Hoebe *et al.,* 2003). TLR4 employs an additional adapter molecule, TRAM which is believed to act as a bridging adapter between TLR4 and TRIF (Rowe *et al*., 2006). TLR4 also signals through MyD88 and this pathway utilises the p65 subunit of NF-κB to mediate activation of the interferon stimulated response element of IFN-β (Smith *et al*., 2005). The induction of type I IFNs by TLR7 and TLR9 depends on MyD88 (Hemmi *et al.,* 2003). Both receptors are intracellular in dendritic cells and are strongly expressed in pDCs (Lee *et al.,* 2003).

In the absence of components of the TLR pathway, other TLR independent pathways were shown to regulate IFN production and antiviral responses (Noppert *et al.,* 2007). The cytoplasmic serine/ threonine kinase, PKR, has been shown to recognise and bind viral dsRNA, and to be involved in the type I IFN response to some viruses (Diebold *et al.,* 2004). This role however, was found to be virus or cell specific (Lopez *et al.,* 2004). Retinoic acid inducible gen-I (RIG-I) was also found to stimulate production of type I IFN in response to dsRNA viral infection (Yoneyama *et al*., 2004). Recently, Watanabe *et al.* (2011) showed that *Helicobacter pylori* causes activation of the nucleotide-binding oligomerization domain(NOD1) signalling pathway, and this induces activation of the type I IFN signalling pathway leading to the generation of Th1 responses (Watanabe *et al.,* 2011).

3.1.5 Functions of type I interferons

3.1.5.1 IFN antiviral mechanisms

Interferons are involved in numerous immune interactions during viral infections and contribute to both induction and regulation of innate and adaptive antiviral immune mechanisms. The essential antiviral role of IFNs was confirmed by the massive increase in susceptibility to virus infection of mice lacking both IFN-α/β and IFN-γ receptors (Knipe *et al*.,2001). All Type I IFNs exhibit strong antiviral activity in target cells due to the induction of antiviral effector proteins such as double-stranded RNA dependent protein kinase (PKR), 2', 5' oligoadenylate, and the large GTPase Mx that promote mRNA degradation, inhibit mRNA translation, induce host cell apoptosis, and/ or inhibit viral RNA polymerase activity (Sen and Ransohoff, 1993; Haller *et al*., 2002).

3.1.5.2 Other immunological functions of type I IFNs

Apart from their antiviral activity type I IFNs have immune functions during the course of non-viral infection (Belardelli, 1995; Bellardelli and Gresser, 1996) such as their role in immunity against parasitic protozoans including *Leishmania major* (Diefenbach *et al.*, 1998) and *Plasmodium falciparum* (Rönnblom *et al.,*1983).

IFN-α/β can work synergistically with other cytokines in many aspects of the immune response. IFN-α/β is also important for the development of a normal splenic architecture, and can affect the function as well as the maturation and differentiation of various dendritic cell types (Fitzgerald-Bocarsly and Feng, 2007). Type I IFN mediates the cross-priming of CD8 T cells and enhances or inhibits Th1 responses depending on the situation (Bogdan *et al.,* 2000; Fitzgerald-Bocarsly and Feng,

2007), and also stimulates the proliferation of activated or memory T cells (Farrar and Murphy, 2000)

When induced together with IL-6, IFN-α/β shows many activities: they activate the differentiation of B cells into plasma cells (Jego *et al.*, 2003), induce cytokine production by NK cells (Nguyen *et al.*, 2002), activate or inactivate macrophages (Jiang and Dhib-Jalbul, 1998) or sensitize macrophages to apoptosis. IFN-α/β also induce the production of other cytokines such as IL-15 with low sensitivity. Fig 4 summarises some of the stimulatory and inhibitory effects of type I IFNs.

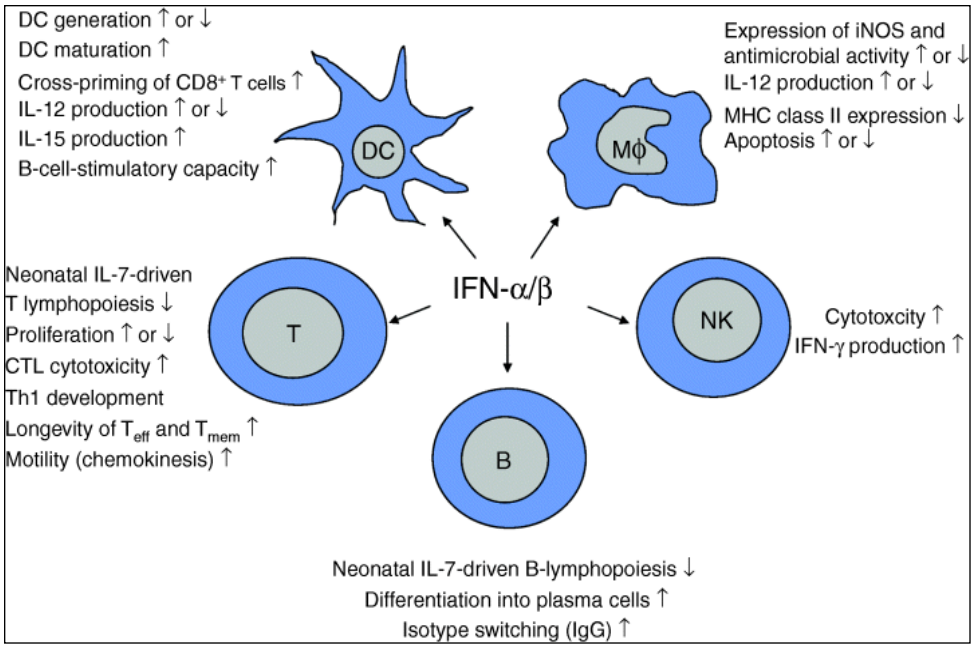

Figure 4. Summary of the stimulatory and inhibitory effects of type I IFNs

Bodgan *et al.,* Immunological Reviews (2004).

Type I IFNs participitate in different immune functions, these include: their effect on the different functions and development of DCs, stimulation of NK cells, activaition and proliferation of T cells, activation or inhibition of macrophages and enhance B cell differentiation into plasma cells.

3.1.5.3 The role of spontaneously released type I IFNs

Although IFN-α/β are released in large quantities upon viral infection, evidence indicates that in the absence of viral or other type I IFN inducers, there is continuous expression of IFN-α/β at very low levels (Bocci, 1985). This minor expression was previously suggested to be important in early antiviral anti-tumor activities and in regulating cell growth (Bocci, 1985; Gresser *et al.,* 1995). More recent studies have shown that the weak signalling by the constantly produced IFN-α/β is important for obtaining rapid optimal expression of IFN-γ and IL-6 (Takaoka *et al.,* 2000).

3.1.6 The role of type I IFNs in non-viral infection

Besides the important antiviral activity for type I IFNs, studies have shown that IFN-α/β also has other functions which are relevant to the pathogenesis or the control of other microbial infections.

3.1.6.1The role of IFN-α/β in diseases caused by protozoan infections

3.1.6.1.1 Leishmaniasis

Leishmania spp. infect mainly macrophages and depending on species leishmaniasis can be a cutaneous or a visceral disease that is fatal if untreated. Murine leishmaniasis was one of the first nonviral infectious diseases in which the mechanisms of action of both endogenous and highly purified exogenous type I IFN were described in detail. In 1970, Herman and Baron observed that in mice infected with *L. donovani*, treatment with poly (I:C) prior to infection caused a significant decrease in the parasite numbers in the liver and also caused a huge rise in IFN-α/β release in the serum (Herman and Baron,1970). As it is well established that type 2

nitric oxide synthase (iNOS) is required for the T_H1-dependent healing of infections with intracellular microbes (Bodgan *et al.,* 2000), the role of iNOS (NOS2) in cutaneous *L. major* infection was studied. iNOS was detectable at the infection site and draining lymph nodes of infected C57BL/6 mice within 24 h of infection. The expression of iNOS was dependent on IFN-α/β and not on IFN-γ (Diefenbach *et al.*, 1998). *In vitro,* significant release of NO was detected when macrophages were stimulated with *L. major* promastigotes together with IFN-α/β (Diefenbach *et al.*, 1998). Prolonged treatment of highly susceptible BALB/c mice with low (but not high) doses of recombinant IFN-β was found to be protective against cutaneous and visceral *L. major* (Mattner *et al.,* 2004). The same study also explained the mechanisms of action of IFN-β in the protective immunity against this parasite, which can be summarized as: 1) enhancing NK cell cytotoxicity; 2) up-regulating IFN-γ and suppression of IL-4; 3) increasing tyrosine phosphorylation of Stat1 and Stat4 (involved in IL-12 signalling); 4) increasing expression of iNOS and 5) restoring sensitivity to IL-12 in the late course of the disease. The treatment decreased the tissue parasite burden and delayed but did not prevent the onset of cutaneous and visceral disease (Mattner *et al.,* 2004). The dose-dependent effect indicates the nature of type I IFN-dependent immune responses can depend on the concentration.

The role of endogenous type I IFN in a South American cutaneous leishmaniasis was recently studied. $IFNAR^{-/-}$ mice were found to develop smaller lesions when infected with *L. amazonesis* in comparison to the wild type mice, indicating a role for type I IFN signalling in promoting leishmaniasis. This was found to correlate with sustained recruitment of neutrophils in $IFNAR^{-/-}$ mice at the early stage of infection confirming a role of neutrophils in innate immunity to this parasite (Xin *et al.,* 2010).

3.1.6.1.2 Trypanosomiasis

The intracellular trypanosome *Trypanosoma cruzi* is the causative pathogen of Chagas disease which in its chronic form results in severe inflammation and organ damage. Control of infection depends largely upon the production of interferon IFN-γ. In infected mice a transient peak of IFN-α was observed at 24h and the daily intraperitoneal treatment of mice with IFN-α/β led to a significant decrease in the number of parasites in the blood (Kierszenbaum and Sonnenfeld, 1982). However, endogenous type I IFN was not required for the control of infection as shown in a study with $IFNAR^{-/-}$ mice as neither the susceptibility to infection nor the production of IFN-γ differed between the knockout or wild type mice (Une *et al*., 2003).

Extracellular trypanosomes of the *Trypanosoma brucei* group cause African sleeping sickness that affects both humans and livestock. Microarray analysis demonstrated that macrophages exposed to the parasite variant surface glycoprotein antigen *in vivo* or *in vitro* expressed type I IFN-dependent genes within 72 h (Lopez et al., 2008). Infected $IFNAR^{-/-}$ mice showed delayed initial control of parasitaemia compared with controls and died sooner. However, $UBp43^{-/-}$ mice that are hyper-responsive to type I IFN were also more susceptible to infection than wild type mice and produced less IFN-γ that is linked to immunity to *T. brucei*. These findings suggested that type I IFN is involved in the early control of infection, but later may down-regulate IFN-γ expression leading to loss of host resistance (Lopez et al., 2008).

3.1.6.1.3Toxoplasmosis

Toxoplasmosis is caused by *Toxoplasma gondii* and is clinically silent in immunocompetent individuals but severe in immunocompromised hosts in which neurological symptoms are common. Infection during pregnancy is an important

cause of abortion in sheep and cattle. During progressive toxoplasmosis of mice, elevated levels of IFN-α/β in the serum correlated with reduced IFN-γ production by splenocytes (Diez *et al*., 1989). Intravenous treatment of mice with 10,000 units of recombinant murine IFN-β protected mice challenged with 100 tachyzoites of *T. gondii* but not mice challenged with 1000 or 10,000 tachyzoites. The protective effect of IFN- β was dependent on IFN- γ production (Orellana *et al.,* 1991).

In vitro results with murine macrophages revealed that neither rate of infection nor replication of *T. gondii* within macrophages was altered by recombinant murine IFN-β. Similarly, when human macrophages were used, no differences in the rates of infection or replication of *T.gondii* in IFN-β treated and control monolayers were detected (Orellana *et al.,* 1991). However the combination of IFN- β and lipopolysaccharide (LPS) was effective in inhibiting the growth of *T. gondii* in monocyte-derived macrophages and this was due to the induction of indoleamine 2,3-dioxygenase (IDO) that catabolises cellular tryptophan required for growth of microbial pathogens (Schmitz *et al*.,1989).

3.1.6.1.4 Malaria

Malaria is caused by different species of *Plasmodium* that infect and destroy red blood cells causing fever, anaemia and (with *P. falciparum*) sometimes death in susceptible adults and young children. Both the innate and adaptive arms of the immune system are involved in immunity to *Plasmodium*. Different studies investigated the involvement of type I IFNs in the immunity to variable species of the parasite. *P. falciparum* has been shown to stimulate human peripheral blood mononuclear cells (PBMC), NK cells and dendritic cells to produce IFN-α (Ronnblom *et al.,* 1983; Ojo-Amaize *et al.*, 1981 and Pichyangkul *et al.,* 2004). IFN-α was also

detected in the sera of mice infected with *P. berghei* or *P. vinckei* (Huang *et al.,* 1968; Clark *et al.,* 1981). Treatment of mice with recombinant IFN-α decreased *P. yoelii* parasitaemia in peripheral blood by inhibiting the production of reticulocytes that this parasite specifically infects (Vigario *et al.,* 2001). This treatment did not affect the pre-erythrocytic stage of infection in the liver.

There is contradiction regarding the role of IFN-α in protective immune responses to malaria. Voisine *et al.,* showed that neither pDCs nor IFN-α/β were essential for parasite clearance as mice depleted of pDCs or IFN-α/β receptor knock-out mice could control *P. chabaudi* infection (Voisine *et al.,* 2010). In contrast, experimental evidence suggested that IFN-α treatment of infected mice had a therapeutic role against the development of cerebral malaria. Treatment with human IFN-α (which mice respond to) prevented death by cerebral malaria in *P. berghei* infected C57BL/6 mice and inhibited the development of the blood-stage of infections at least in part by increasing IFN-γ levels in the blood (Vigario *et al.,.* 2007).

Recently, two studies highlighted the possible role of type I IFNs in immunity to *P. falciparum.* Using microarray analysis, the expression profiling of PBMCs derived from patients with *P. falciparum* malaria detected elevated expression of interferon-inducible genes (ISGs). The study furthermore showed that PBMCs stimulated with schizont-infected RBCs induce IFN-α at the protein level and IFN-β mRNA (Sharma *et al.,* 2011). In addition it was found that parasites upregulated expression of IFN-α/β-dependent genes (Grangeiro de Carvalho *et al*., 2011).

3.1.6.2 The role of IFN-α/β in bacterial infections

3.1.6.2.1 Intracellular pathogens

3.1.6.2.1.1*Chlamydia*

Different studies have indicated an *in vitro* inhibitory effect of type IFNs on infection with different species of *Chlamydia,* the earliest going back to 1963 (Suetlenfuss and Pollard, 1963; Hanna *et al.,* 1966). *Chlamydia* was also capable of causing different host cells to produce type I IFNs (Rodel *et al.,* 1998). Rothfuchs *et al.* showed that type I IFNs are beneficial to in vitro cultured mouse bone marrow derived macrophages in resisting *C. pneumonia* infection through enhancing IFN-γ and NO production (Rothfuchs *et al*., 2001). However, in an *in vivo* study, type I IFNs were shown, instead of being protective, to promote *C. muridarum* infection. $IFNAR^{-/-}$ mice suffered less body weight loss, lower organism burden, and milder pathological changes in the lung than WT mice following respiratory tract *C. muridarum* infection. There was greater infiltration of macrophages to the lung and less apoptosis of the infiltrating macrophages in $IFNAR^{-/-}$mice than WT mice (Qiu *et al.,* 2008).

3.1.6.2.1.2 *Listeria*

Although a previous study highlighted a protective role for IFN-β in the immunity of mice against *Listeria monocytogenes* (Fujiki and Tanaka, 1988), further studies indicated that type I IFN signalling actually enhanced the infection with *L. monocytogenes.* $IFNAR^{-/-}$ mice developed a much lower titre of infection in their liver and spleen when compared to wild type mice (Auerbuch *et al*., 2004). After a non-fatal dose of *Listeria* was given*,* treatment with poly (I:C) induced mortality in wild type mice but not in $IFNAR^{-/-}$ mice (O'Connell *et al.,*. 2004). Until recently the

mechanisms underlying these effects were not completely understood but it is thought that they may be related to the pro-apoptotic effects of *Listeria*-induced type I IFN (Carrero *et al.,* 2004). Rayamajhi *et al.* have recently described the possible mechanism of type I IFN action. Cultured macrophages infected with *L. monocytogenes* responded poorly to IFN-γ treatment due to the down-regulation of IFNGR. The low expression of IFNGR was shown to be associated with the production of IFN-α/β by infected cells (Rayamajhi *et al.*, 2010).

3.1.6.2.1.3 *Mycobacterium*

Infection with *Mycobacterium tuberculosis* stimulated human macrophages and dendritic cells to produce type I IFN, and this was required for the expression of the chemokine CXCL10, a known activator of NK cells and T cells (Lande *et al.,* 2003).

The ability of *M. tuberculosis* strains to induce type I IFN production has been found to correlate with bacterial virulence (Manca *et al*., 2001). In comparison to the WT animals, the *M. tuberculosis*–infected IFNAR$^{-/-}$mice had a lower rate of mortality and survived the infection for longer (Manca *et al*., 2005); however, in the first 2–3 months after infection, their bacterial load was similar in the lungs and slightly lower in the spleen (Cooper *et al*., 2000; Manca *et al.,* 2005; Stanley et al., 2007).

In addition, type I IFN produced after intranasal application of poly (I:C) stabilized by encapsulation in poly-l-lysine and carboxymethylcellulose exacerbated tuberculosis in wild type mice but not IFNAR$^{-/-}$. The mechanism of this was found to involve recruiting a population of CCR2-expressing macrophages that is highly tolerant of *M. tuberculosis* proliferation (Antonelli *et al*., 2010).

3.1.6.2.1.4 Invasive enteric bacteria

Studies also indicated a protective role for type I IFNs in immunity against enteric bacteria including *Shigella* and *Salmonella.* Peripheral blood cells were able to produce IFN-α after stimulation with *Shigella flexneri* or *Salmonella (enterica) typhimurium* (Klimpel *et al.*, 1988). In an *in vitro* study, pretreatment of cell monolayers with natural and recombinant IFNs reduced the number of *Shigella*-infected cells (Niesle *et al*., 1986). Treatment of neonatal mice with mouse fibroblast interferon significantly decreased mortality due to *S. typhimurium* in a dose dependent manner (Bukholm *et al.,* 1984). Both MyD88 and IFN-α/β were shown to be important for dendritic cell activation of T cells mediated by *S. typhimurium* (Tam *et al*., 2008).

3.1.6.2.2 Extracellular pathogens

Type I IFN signalling was found to be fundamental to host defense against infection with extracellular bacteria including group B streptococci (GBS), *Streptococcus pneumoniae* and encapsulated *Escherichia coli.* This was confirmed by a marked reduction of survival in $IFNAR^{-/-}$ mice while wild type survived the infection (Mancuso *et al*., 2007). In the case of GBS infection, wild type mice survived whereas there were mortalities among $IFNAR^{-/-}$ and IFN-$\beta^{-/-}$ mice. There were fewer deaths in the group of IFN-$\gamma R^{-/-}$ mice than in $IFNAR^{-/-}$ mice and additive mortality was observed in animals lacking both receptors (Mancuso *et al*., 2007). Additionally, in the absence of IFN-α/β signalling, a significant reduction in macrophage production of IFN-γ, NO and TNF-α was observed after stimulation with GBS (Mancuso *et al*., 2007).

3.1.6.3 Infection with helminthes

Few studies are reported in the literature concerning the role of type I IFNs in immunity to helminthic infection. *Shistosoma mansoni* eggs (but not the larval stage) were found to activate myeloid dendritic cells for the expression of different proinflammatory cytokines including IFN-β and various IFN-inducible genes. This was completely dependent on IFN-α/β receptor signalling (Trottein *et al.,* 2004). Also, IFN-α2 treatment of mice infected with *Echinococcus multilocularis* resulted in 75% protection against intrahepatic lesions and about 50% against infection (Godot *et al*., 2003).

3.1.6.4 Fungal infections

A good example of a model used to study the role of type I IFNs in fungal infection involved the pathogenic yeast *Cryptococcus neoformans.* $IFNAR^{-/-}$ mice showed higher fungal burden, increased eosinophilic lung infiltrates and significant production of Th2 cytokines such as IL-13, IL-4 and IL-5, when compared to wild type mice (Biondo *et al*., 2008). It was also shown that *C. neoformans* induced IFN-β gene expression in macrophages and dendritic cells. However, a previous study with *Candida albicans* indicated an opposing role for type I IFNs in immunity against fungal infection. Treatment of SCID mice with poly (I:C) significantly enhanced susceptibility to systemic candidiasis. This was partially reversed by administering to (I:C)-treated mice either anti-IFN-γ or anti-IFN-α/β (Jensen *et al.,*1992). In the case of *Aspergillus fumigates* (a mold), treatment of wild type or athymic Swiss mice with IFN-α/β or poly (I:C) significantly decreased mortality (Maheshwari *et al.,*1988).

3.1.7 IFN-γ in viral and microbial infections

Like type I IFNs, IFN-γ has antiviral activity and plays a role in the long-term protection against many viral infections. Among these viruses are hepatitis B, herpes simplex, lymphocytic choriomeningitis and mouse pox virus (Muller *et al.,* 1994; Cantin *et al.,* 1999 and Steed *et al.,* 2006).

IFN-γ plays a crucial role in host immunity against different intracellular pathogens including *Mycobacterium tuberculosis* (Flynn *et al.,* 1995), *Salmonella typhimurium* (Masteroni *et al.,* 1999), *Listeria monocytogenes* (Fehr *et al.,* 1997), and *Toxoplasma gondii* (Ceravolo *et al.*, 1999; Suzuki *et al.,* 2011). The role of IFN-γ in both innate and adaptive immunity to *C. parvum* is explained in depth in Chapter One of this thesis.

3.1.8 Clinical applications of IFNs

The range of biological functions exhibited by IFN- α and IFN-β make these cytokines promising agents in the development of treatments for various diseases.

The first approval to use human IFN-α2 for therapy was made by the U.S.A Food and Drug Administration in 1986 for the treatment of hairy cell leukemia. Recombinant IFN-α was found to be effective in the treatment of other malignancies including chronic myeloid leukemia (CML), B and T cell lymphomas, melanomas and Kaposi's sarcoma (Parmer and Platanias, 2003). Some data also showed that IFN-α gave some benefit when used in drug combination regimens or as an adjuvant in a subset of patients with multiple myeloma (Osterborg *et al.,*1993). Type I IFNs have direct effects on tumor cells but also act on immune cells that play a part in antitumor response (Ferrantini *et al.,* 2007). The fact that IFN-α can induce the differentiation and the activation of dendritic cells may be relevant for the use of IFN-α in the

development of cancer vaccines (Ferrantini *et al.,* 2007). IFN-α is also approved for the treatment of hepatitis B and C (D'Souza and Foster, 2004b; D'Souza and Foster 2004a) and for the treatment of genital warts. In addition, human primary immunodeficiencies of type I IFNs have been described. This is thought to be due to defects in either type I production or signalling (Casrouge *et al.,* 2006). Affected patients were found to be highly susceptible to viral infections and they were shown to be good candidates for treatment with type IFNs (Casrouge *et al.,* 2006). IFN-β is approved for the treatment of relapsing-remitting multiple sclerosis, (Tourbah and Lyon-Caen, 2007).

IFN-γ is used as a prophylactic agent in patients with chronic granulomatous disease and in patients with defective IFN-γ production. Some data are available about the use of IFN-γ in the treatment of malignant osteropetrosis (Madyastha, 2000). Although many studies indicate that IFN-γ has a considerable effect in the treatment of non-viral infections such as parasitic diseases (Murray, 2005), its clinical application has not been attempted (Chelbi-Alix and Wietzerbin, 2007).

3.1.9 Hypothesis and aims

Findings on the part played by IFN-α/β in immunity to nonviral intracellular microbial pathogens, including protozoa, is contradictory ie type I IFNs play a significant role in protective immunity in some cases but may inhibit immunity in other cases. The part played by type I IFN in immunity to intestinal apicomplexans such as *Cryptosporidium* has been unknown.

In this chapter it is hypothesised that type I IFNs play an important role in the innate immunity to *C. parvum.*

Therefore the aims were:

1. To study the effect of type IFNs (IFN-α and IFN-β) on the development of *C. parvum* infection of enterocytes.
2. To investigate the effect of type IFNs on *C. parvum* infection of immunocompetent and immunocompromised mice.
3. To examine possible mechanisms of action of type I IFNs both *in vitro* and *in vivo.*

3.2 MATERIALS AND METHODS

3.2.1 Reagents

Human IFN-α2 was supplied by Roche, murine recombinant IFN-α4 and IFN-β were supplied by Serotec. Ferrous sulphate ($FeSO_4$), tryptophan and guanidino-*N*-methylated *L*-arginine (N^G-MMA) were obtained from Sigma-Aldrich and were used to study the possible mechanisms of IFN-α antimicrobial activity. Polyinosinic-polycytidylic acid or poly (I:C) and *Salmonella typhimurium* lipopolysaccharide were supplied by Sigma-Aldrich. Anti-mouse CD11b, CD11c and MHC class II fluorochrome-conjugated antibodies for flow cytometry were obtained from BD Pharmingen.

3.2.2 Type I IFN neutralizing antibody

Anti-IFN-α/β serum was a gift from Dr Michael Tovey (Institute Andre Lwoff, Villegiuit, France) and was produced in sheep hyperimmunized with highly purified IFN-α/β (Gresser *et al.,* 1976). The neutralizing titre expressed as Tenfold Reduction Units was 3.2×10^5 U/ml and each mouse received 100µl. Control mice received normal sheep serum diluted to the same extent.

3.2.3 Tissue culture and infection

As described in Chapter 2, Caco-2 and CMT-93 were grown in 6 or 24-well plates with or without coverslips. The cells were then infected with *C.parvum* by addition of oocysts. In experiments studying the effect of IFN-α or -β on parasite development, the cell monolayers were incubated with the cytokine 24h prior to infection alone or together with other reagents to study the possible mechanisms of IFN activity.

3.2.4 Antiviral assay

Supernatants from infected CMT-93 cells were collected and added to murine fibroblast L929 monolayers cultured in a 96 wells. As described in General Materials and Methods, murine L929 fibroblasts were cultured in a 96 well plate for 24 h then were either co-cultured for another 24 h with supernatants collected from previously infected CMT-93 cells or with standards of recombinant murine IFN-α (most of the cytokine produced would be IFN-α). Subsequently the cells were infected with encephalomyocarditis virus (ECMV) and viral plaque formation was examined after 16h incubation at 37C°. At the end of the incubation period the cells were fixed with 4% paraformaldehyde in PBS and stained with 0.25% solution crystal violet to show the extent of plaque formation. Absorbance was then red at 580nm and the anti-viral activity (inversely related to viral damage of cell monolayer) of supernatants was measured in comparison to that of the recombinant IFN-α standards.

3.2.5 ELISA for IFN-α

Supernatants from infected CMT-93 cells were collected 24 h post-infection and the amount of IFN-α was measured by ELISA using a mouse IFN-α ELISA kit (PBL Biomedical Laboratories). The assay was carried out as instructed by the manufacturer. The ELISA plate had wells pre-coated with anti-mouse IFN-α capture antibody (host of antibody not divulged). Following a period of 1 h incubation of 100 µl standards or supernatants samples at room temperature, the wells were washed using cold washing solution (supplied with kit) and tapped dry, then were incubated for 24 h with 100 µl of detection antibody solution (biotinylated and supplied with kit). After that the contents of the plate were emptied and the plate was washed and dried. The wells were then incubated for 1h with 100 µl horseradish peroxidase (HRP) conjugated avidin solution (supplied), washed and further incubated in the

dark for 15 minutes with 100 µl of tetramethylbenzidine (TMB) substrate solution. The reaction was then stopped by supplied stop buffer and the amount of production of IFN-α was determined after measuring optical densities using a microplate spectrophotometer at 450nm.

3.2.6 Generation of dendritic cells

This technique was performed in collaboration with Dr Daniel Korbel and the protocol used followed the one demonstrated by Maroof (2001). Femurs and tibias of adult SCID mice were flushed with PBS to obtain the bone marrow. Erythrocytes were lysed using 0.83% ammonium chloride and the single cell suspensions of bone marrow in complete RPMI1640 medium were incubated in plastic Petri dishes overnight at 37°C. Non-adherent cells were removed and fresh complete medium with 20ng/ml granulocyte macrophage-colony stimulating factor (GM-CSF) and 100U/ml IL-4 was added. On day 3 the medium was changed and dendritic cells were collected on day 7. The purity of the cells was confirmed by microscopy and flow cytometry using anti-mouse CD11b (to detect any macrophage contamination), CD11c and MHC class II antibodies.

3.2.7 RNA extraction and real-time quantitative PCR

RNA extraction and reverse transcription to cDNA from CMT-93 cells, bone marrow-derived cells and small intestine tissue samples was performed as described in Chapter 2. Amplification and relative quantification of mRNA expression levels were performed using the Delta-Delta-Ct (ΔΔCt) method. The primer sequences were as follows: murine β-actin forward 5_-CCT TCC TTC TTG GGT ATG GAA T-3_ and reverse 5_-GCACTGTGTTGGCATA GAGGT-3_ (106 base-pairs [bp]); murine IFN-γ forward 5_- GCC AAG TTT GAG GTC AAC AAC-3_ and reverse 5_-ATC AGC AGC

GAC TCC TTT TC-3_ (121 bp); murine IFN-α forward 5_-CTG CTG GCT GTG AGG ACA TA-3_ and reverse 5_-GGC TCT CCA GAC TTC TGC TCT-3_ (105 bp); and murine IFN-β forward 5_-GCA CTG GGT GGA ATG AGA CT-3_and reverse 5_-AGT GGA GAG CAG TTG AGG ACA-3_ (135 bp). The IFN-α sequences are a full match for IFN-α4, IFN-α6, and IFN- α7 and a single base mismatch for IFN-α1, IFN-α2, IFN-α5, and IFN-α11.

3.2.8 Animal infection and parasite counting

As described in Chapter 2 (Materials and Methods) BALB/C or SCID mice were infected using 1 x10^4 oocysts *C. parvum* and the level of infection was assessed microscopically by counting the parasite numbers in acid-fast stained stool smears.

Other experiments investigated the early stages of infection of neonatal mice. Seven-day old mice were infected with 1x10^5 oocysts and the animals were sacrificed 48h post-infection. As it was an early stage of infection when few oocysts are likely to have yet formed infection was measured histologically. The ileum was removed and placed in formal saline fixing solution. The tissue was embedded in paraffin and 5μm longitudinal sections prepared and stained with haematoxylin and eosin and the infection score was determined semi-quantitatively by counting parasites in intestinal villi. Ten villi were selected at random and given an infection score of 0-10 (0, no parasites; 1, up to 10% of cells infected; etc. up to 10, all cells infected). A total score (highest possible being 100) was obtained for each mouse and the mean score calculated for the group.

3.3 RESULTS

3.3.1 *In vitro* studies

3.3.1.1 Infection of intestinal cell lines with *C. parvum*

As described in Chapter 2 (Materials and Methods) human Caco-2 or murine CMT-93 cells were grown on glass cover slips in a 24-well plate before infection with *C.parvum* oocysts. After the period of 24h of infection, cell monolayers were washed with PBS, fixed and stained with Giemsa stain and the infection level was measured microscopically by counting parasites in 20 random fields under X1000 magnification with oil-immersion. Fig 5 shows *C. parvum* infection of CMT-93 cells.

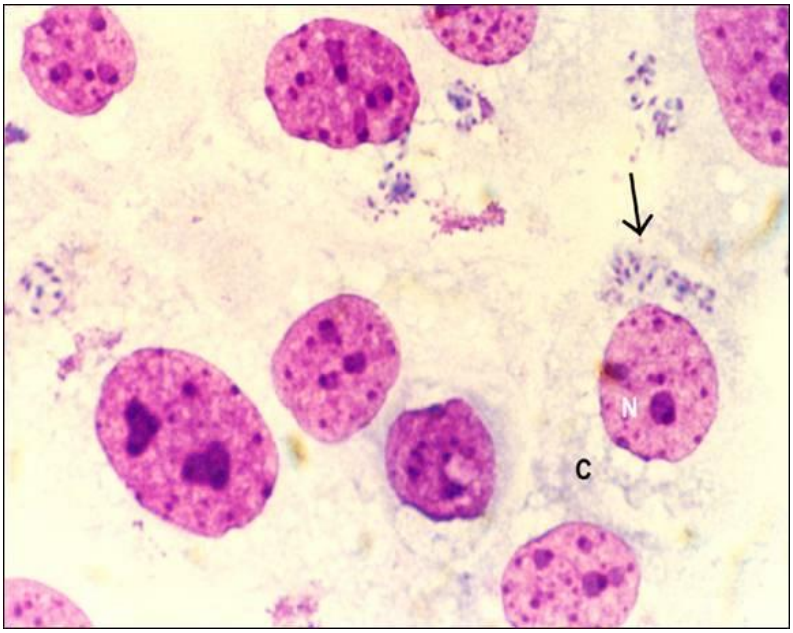

Figure 5.*C. parvum* infection of CMT-93 cells.

CMT-93 cells were infected for 24 h, fixed and stained with Giemsa stain. Parasite numbers were counted microscopically. N=nucleus, C=cytoplasm, mature meront arrowed.

3.3.1.2 The effect of IFN-α on parasite development in *C. parvum*-infected Caco-2 cells

To determine if IFN-α has an inhibitory role on infection with *C. parvum*, human Caco-2 cells were incubated with different concentrations of human IFN-α2 (1, 10 or 100 IU/ml) 24 h prior to infection. The level of infection was measured by counting the parasites microscopically 24 h postinfection. There was a significant decrease in the number of parasites in the cells which were treated with the cytokine and this effect was dose dependent (Fig 6; $p < 0.0012$).

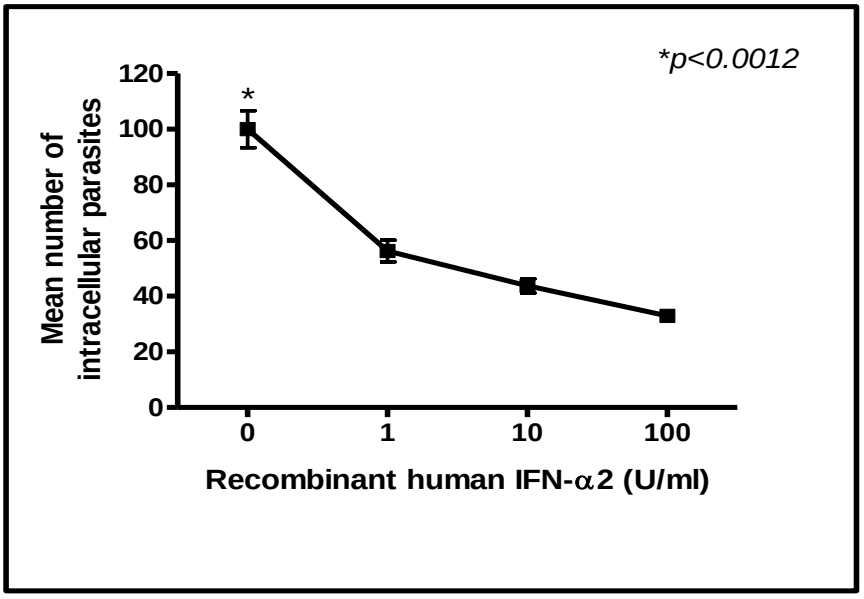

Figure 6.The effect of varying concentrations of IFN-α2 on development of *C. parvum* in Caco-2 cells.

Since an inhibitory effect of IFN-α was found with the lowest concentration of 1 U/ml, the effect of even lower concentrations (0.01 and 0.1 U) of the cytokine was measured. A dose of 0.1 U/ml was effective in inhibiting infection ($p<0.004$), but a dose of 0.01 U/ml had no effect *(*Fig 7. *p<0*.551).

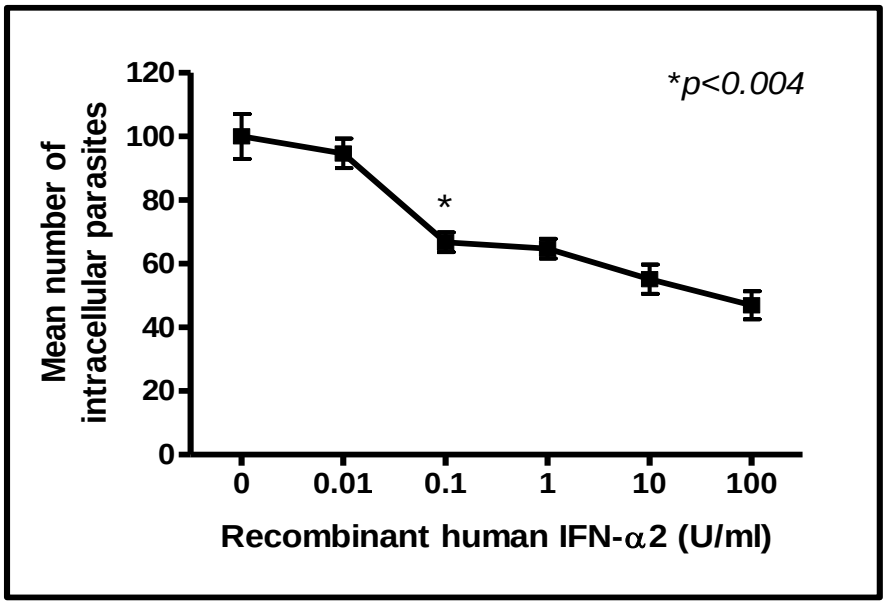

Figure 7.The effect of lower concentrations of IFN-α2 on *C. parvum* infection of Caco-2 cells.

3.3.1.3 The effect of murine IFN-α4 on parasite development in infected in CMT-93 cells

To study whether the anti-parasitic activity of IFN-α is general and not specific for human cell lines, the effect of murine IFN-α4 on infection of murine CMT-93 cells was examined. As in the previous experiments, the cells were treated with different concentrations of murine IFN-α4 and the subsequent level of infection was measured.

It was found that the cytokine had a significant inhibitory effect on parasite development in CMT-93 cells (p<0.0001). However there was no significant increase in the inhibitory activity with cytokine concentrations higher than 1U/ml (Fig 8; p<0.06).

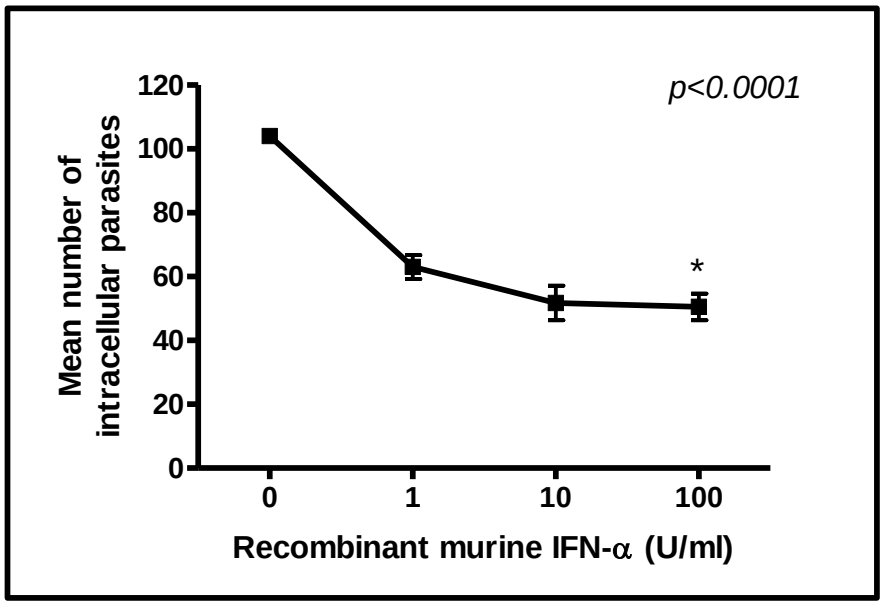

Figure 8.The effect of IFN-α4 on parasite development in CMT-93 cells.

3.3.1.4 Effect of IFN-α2 on the very early parasite development

Previous *in vitro* studies have shown that *C. parvum* sporozoites need 2-3 h to invade the epithelial cells. The possible effect of IFN-α2 on the very early stage of *C. parvum* development was examined. Caco-2 cell monolayers were treated with 10 or 100U/ml of the cytokine for 24 h, then were infected for 3 h only. The cells were then washed, fixed, stained and the level of infection was measured.

Interestingly, it was found that as early as 3h post infection IFN-α pre-treatment had caused a significant reduction in numbers of intracellular parasites (*p<0.003*). This may mean that IFN-α inhibits *C. parvum* infection at least partially by preventing the parasite invading the cell. Results are summarised in Fig 9.

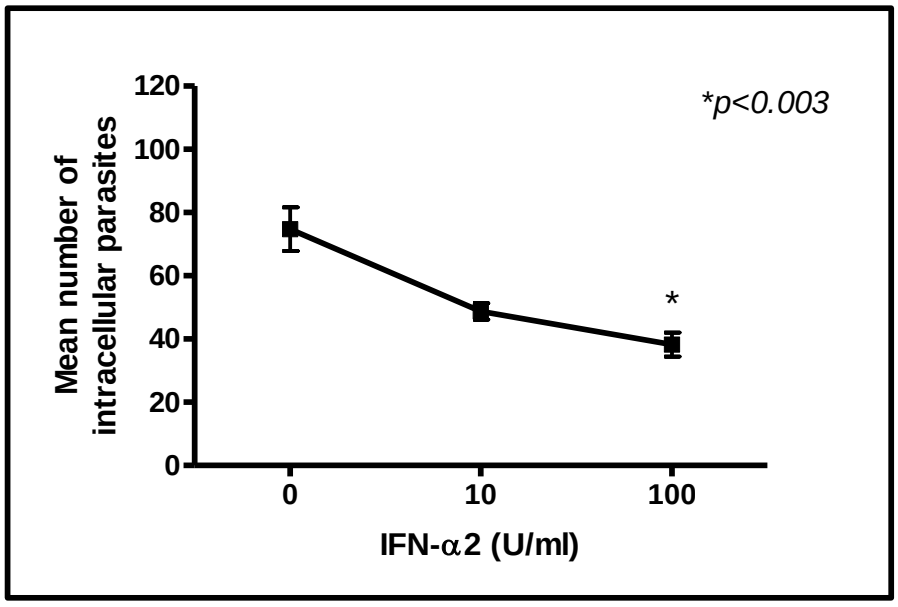

Figure 9. The effect of IFN-α2 pre-treatment on *C. parvum* development in Caco-2 cells 3h after addition of oocysts to cells.

3.3.1.5 The effect of IFN-α on different temporal and developmental stages of *C. parvum* during infection

To investigate which developmental stages of parasite growth the inhibition of development by IFN-α may occur, the level of infection of cells was examined at 3h (normally mostly trophozoites), 8h (increasing numbers of developing meronts) and 24h (normally developing and mature meronts) postinfection in cytokine pre-treated or control cells.

The cells were initially treated with 100 U/ml of IFN-α2 for 24 h prior to infection then the subsequent infection was measured at three different times. As summarised in Fig 10, similar to the results of the previous experiment, at 3h postinfection the level of infection was significantly reduced (about 50%) in the cells treated with the cytokine in comparison with control cells. However at later times the relative number of parasites did not decrease further.

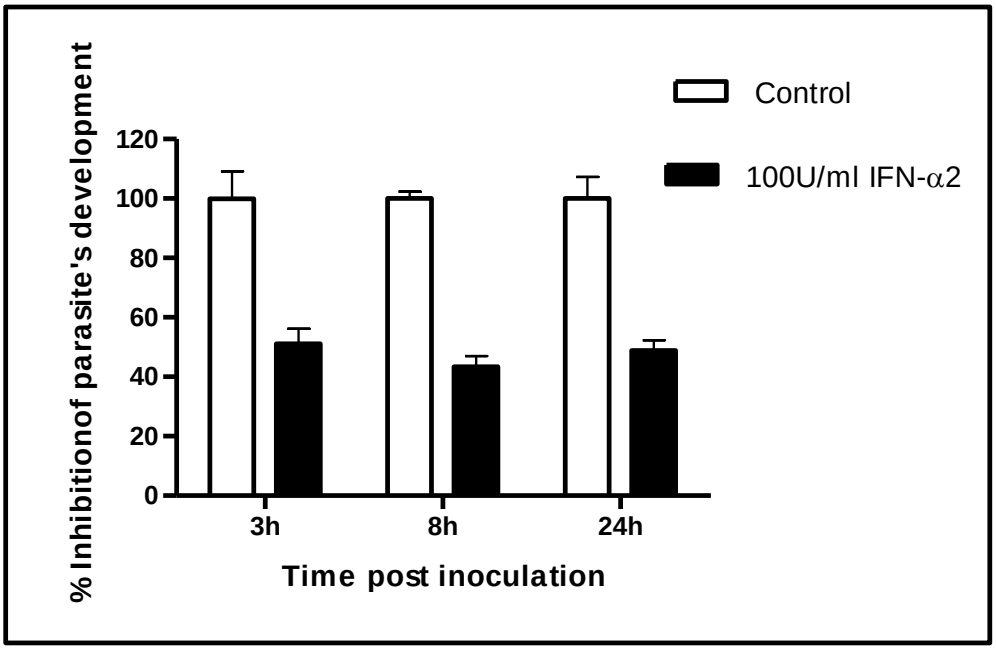

Figure 10. The effect of IFN-α 2 on intracellular *C. parvum* development in Caco- 2 cells, 3, 8, 24h post infection. About 50% inhibition of parasite development was observed 3h postinfection, but no further increase in the level of inhibition was observed later.

Development of some first generation of meronts of *C. parvumin vitro* is well under way at 8h postinfection. As summarised in Table 4, the percentages of small, growing and maturing intracellular parasites in the IFN-α 2-treated and the control samples were also assessed at this stage of infection in this experiment. IFN-α did not appear to have any effect on the rate of maturation of the parasites up to 8h. This observation suggests that parasites once established intracellularly in IFN-α-treated cells develop normally.

Treatment	CON	IFN-α	CON	IFN-α	CON	IFN-α
Development stage	Trophozoites (single nucleus)		Immature meronts (relatively small with 2 nuclei)		Large meronts (large with 4-8 nuclei)	
Percentage of parasite population	34.3±5.6%	38± 3.4%	58± 2%	58.6± 3%	8± 5.3%	3.3± 1%

Table 4. The effect of IFN-α2 on the maturation of *C. parvum* in Caco-2 cells examined at 8h postinfection.

3.3.1.6 Other possible mechanism(s) of action of IFN-α

The previous experiment suggested that IFN-α inhibits the parasite invasion of cells. Certain other antimicrobial mechanisms have been associated with type I and type II IFN activity against different intracellular microbial pathogens. The following experiments studied other antimicrobial mechanisms that may partially explain the inhibitory function of IFN-α on *C. parvum* development.

3.3.1.6.1 The effect of iron (Fe^{2+}) on the inhibitory action of IFN-α2 of *C. parvum* development

It is well known that Fe^{2+} is an important component required for the development of many microorganisms including *Cryptosporidium* and in this laboratory depletion of intracellular Fe^{2+} was found to be one of the IFN-γ-mediated antimicrobial activities against *C.parvum* (Pollok *et al.*, 2001). It was therefore necessary to check if IFN-α also inhibits parasite development by depleting cellular Fe^{2+}.

To study this possible mechanism, Caco-2 cells were cultured with 10 or 100 U/ml of IFN-α, then infected with or without 200μM $FeSO_4$. The concentration of $FeSO_4$ used in this experiment was found to prevent the killing activity of IFN-γ on *C. parvum* (Pollok *et al.*, 2001). The cells were infected with *C. parvum* in the absence or presence of $FeSO_4$ for 24h.

Fig 11 shows that $FeSO_4$ alone had no effect on the infection when compared to infected controls (p<0.354*)*. In addition, $FeSO_4$ did not affect the inhibitory action of IFN-α2 when compared to the cells which were treated with the cytokine and had no $FeSO_4$ added (p<0.1904*)*.

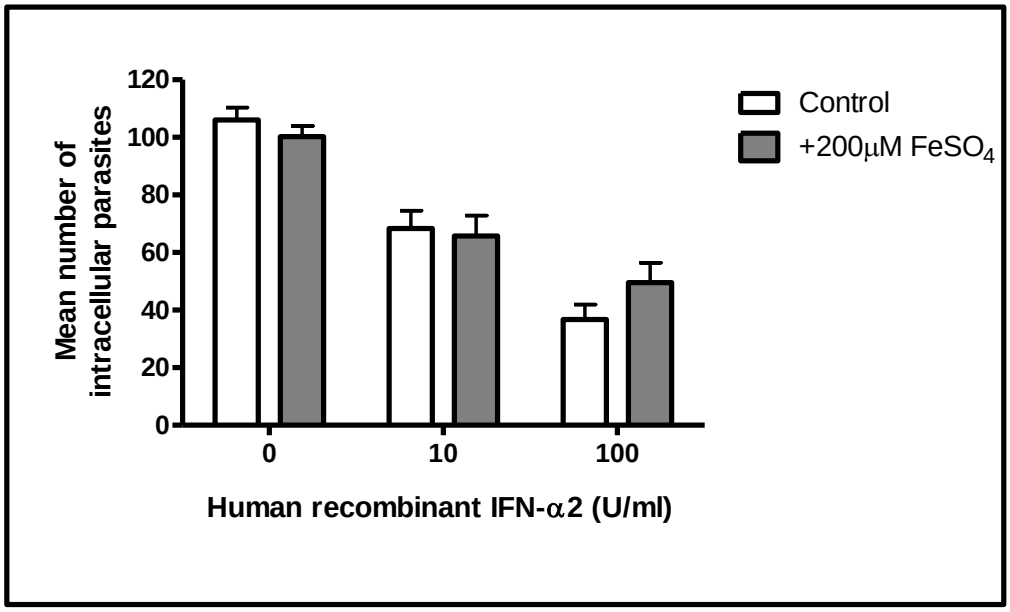

Figure 11.The effect of FeSO4 on the inhibitory action of IFN-α2 in *C. parvum* development. Caco-2 were incubated with IFN-α2 and then infected in the presence or absence of 200 μM FeSO4 and level of infection was measured 24h later. FeSO4 had no significant effect on the inhibitory action of IFN-α2 (p<0.1904).

3.3.1.6.2 The effect of tryptophan on the action of IFN-α2 on *C. parvum* development

L-tryptophan is an essential amino acid that is required by many microbial pathogens for their growth. Cellular depletion of L-tryptophan may help the host to resist the development of different pathogens (King and Thomas, 2007). Indoleamine 2,3 dioxygenase (IDO) is an enzyme that depletes L-tryptophan from the tissue microenvironment by oxidative degradation in the kynurenine pathway. The IDO gene is only induced in response to certain inflammatory stimuli, notably IFN-γ, and to a lesser degree by IFN-α, IFN-β and also lipopolysaccharide (LPS). Therefore the effect of a high concentration of exogenous tryptophan on the action of IFN-α was investigated.

As in the previous experiment Caco-2 cells were initially treated with IFN-α with or without 250 µg/ml of tryptophan, then infected for 24 h in the presence or absence of tryptophan prior to measuring the infection level. In this laboratory, a similar concentration of tryptophan was found to prevent IFN-γ-induced killing of *Encephalitozoon intestinalis* in CMT-93 cells. (Choudhry *et al*., 2009). Fig 12 shows that exogenous tryptophan had no effect on *C. parvum* development when used on its own (p<0.712); also, it had no effect on IFN-α-mediated inhibition of *C. parvum* growth (p<0.45).

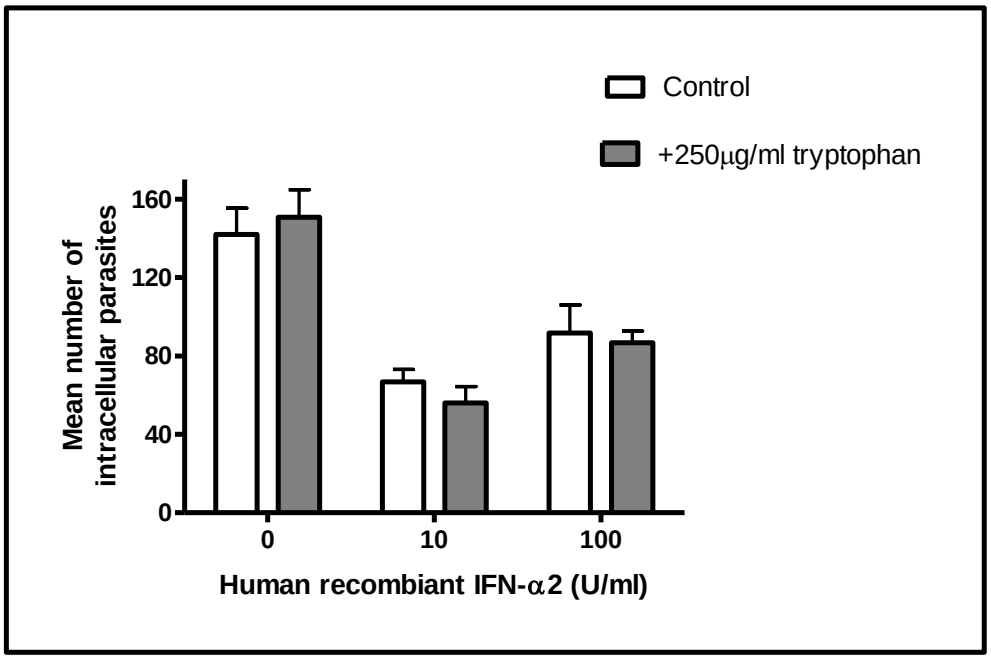

Figure 12.The effect of exogenous tryptophan on the anti-microbial activity of IFN-α2. Caco-2 cells were incubated with different concentrations of IFN-α with or without tryptophan and the level of *C. parvum* infection assessed microscopically. Treatment with tryptophan had no significant effect on IFN-α mediated growth inhibition (*p*<0.45*).*

3.3.1.6.3 The effect of an inducible nitric oxidesynthase (iNOS) inhibitor on IFN-α action

The NOS inhibitor N^G-MMA, acts by blocking access of arginine to the enzyme and so preventing nitric oxide production. Hence to investigate whether N^G-MMA had any effect on the action of IFN-α against infection, Caco-2 cells were pre-treated with the cytokine, infected in the presence or absence of 500μM N^G-MMA and the infection was examined as described previously. The dose of N^G-MMA used in this study was found previously to block IFN-γ activity against *Toxoplasma gondii* in rodent enterocytes (Dimier *et al.,* 1998). No significant difference in the inhibitory action of

IFN-α2 was observed when used in combination with the NOS inhibitor (p<1) and no significant effect on infection was found with N^G-MMA (Fig 13; $p<0.783$).

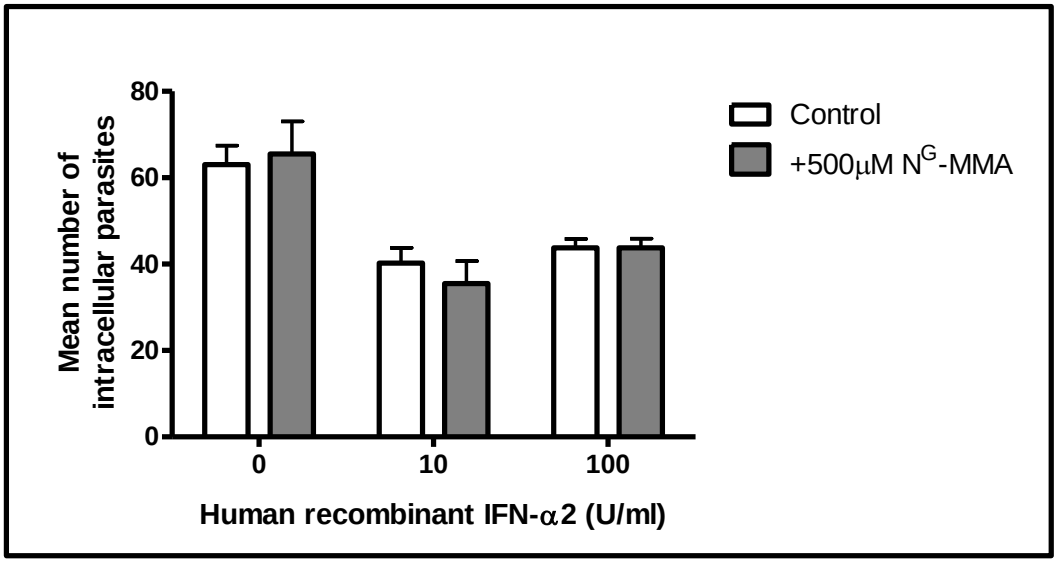

Figure 13.The effect of iNOS inhibitor NG-MMA on IFN-α2 on inhibition of *C. parvum* development in Caco-2 cells. Caco-2 cells were incubated with IFN-α2 and treated with or without 500μM N^G-MMA during infection and the numbers of parasites assessed microscopically 24h postinfection. N^G-MMA had no significant effect on the inhibitory action of IFN-α2 (p<1).

3.3.1.7 The effect of IFN-β on *C. parvum* infection of CMT-93 cells.

It was of interest to investigate the effect of another type of type I IFN, IFN-β, on *C. parvum* development in enterocytes. As in the experiments with IFN-α, CMT-93 cells were treated with different concentrations of IFN-β (1000, 100,10 or 1 U/ml) 24 h prior to infection and the level of infection was measured after 24 h. As summarised in Fig 14, similar to what was found with IFN-α, there was a significant reduction (about 60%) in the level of infection in the cells treated with 1U/ml of IFN-β in comparison to the untreated cells (p <0.0025) but no further inhibitory activity was observed with higher concentrations.

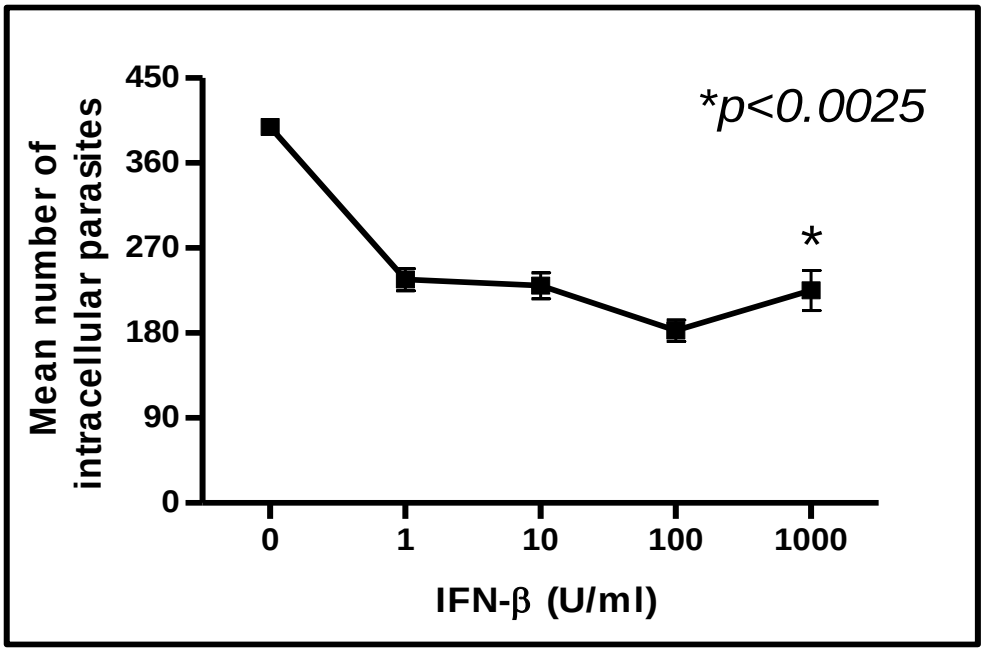

Figure 14.The effect of varying concentrations of IFN-β on *C. parvum* development in CMT-93 cells.

3.3.1.8 The effect of TLR3 agonist poly (I:C) on *C. parvum* infection *in vitro*

The TLR3 agonist polyriboinosinic:polyribocytidylic acid (poly (I:C)) is a synthetic dsRNA described to be a potent inducer of type I IFN (Field *et al.,*1967). Studies by others indicated treatment of epithelial cells with poly (I:C) enhanced the antiviral activity of the cells (Schaefer *et al.,* 2005). So to investigate if the epithelial cells are capable of producing type I IFN that inhibits infection with *C. parvum*, enterocytes were pre-treated with poly (I:C) before infection.

CMT-93 cells were grown on cover slips as in previous experiments, and then cultured for 24 h with different concentrations of poly (I:C) (0.025, 0.25, 2.5 or 25 µg/ml). Then, the cell monolayers were infected with *C. parvum* for 24h. The level of infection was measured by counting the parasites microscopically. As shown in Fig

15, the results indicated that the level of infection was significantly reduced by treatment with poly (I:C) and this effect was dose dependent (p<0.0002).

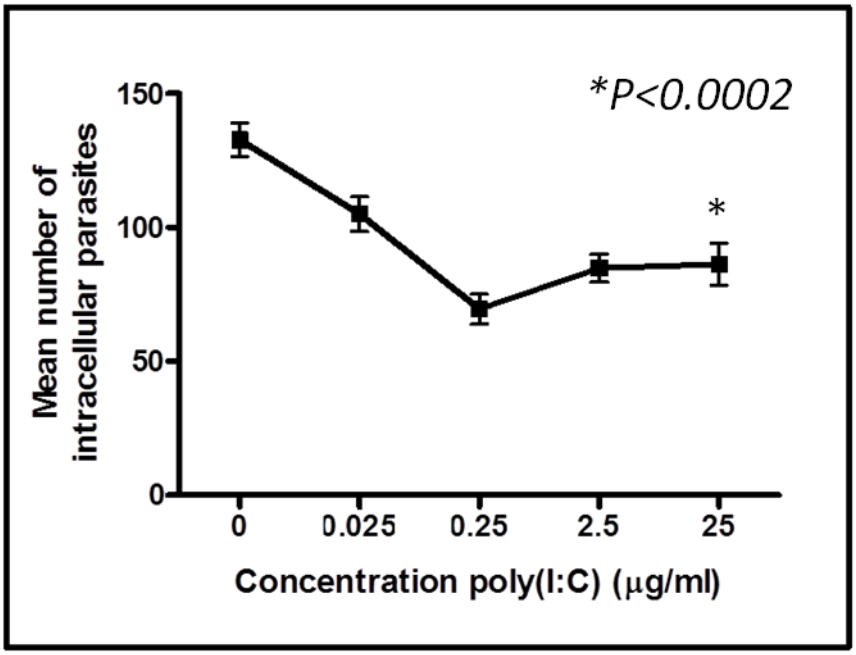

Figure 15. The effect of different concentrations of poly (I:C) on *C. parvum* development in CMT-93 cells.

3.3.1.9 Antiviral assay to investigate the production of type I IFN by infected enterocytes

The previous result showed that poly (I:C)-stimulated enterocytes can inhibit infection by *C. parvum,* potentially by producing type I IFN. It was necessary to prove that type I IFN was produced, however. As in the previous experiment CMT-93 cells were stimulated with poly (I:C) prior to infection with *C. parvum*. Culture supernatants from infected and control samples were collected 24 h later and used to test for the production of type I IFN.

The amount of released type I IFN was measured indirectly using a bioassay of antiviral activity, recruiting the murine fibroblast cell line L929 infected with EMCV.

Interestingly, it was shown that supernatants derived from infected CMT-93 alone had a significant antiviral activity which was not the case in the supernatants from uninfected cells (Fig 16; $p < 0.026$).

Poly (I:C) on its own stimulated CMT-93 cells to produce type I IFN. When there was both infection and poly (I:C) treatment, the level of type I IFN produced was greater than either treatment alone with 2.5 and 25 µg/ml poly (I:C). These results indicate that enterocytes are capable of the release of type IFN after infection with *C. parvum* or treatment with poly (I:C).

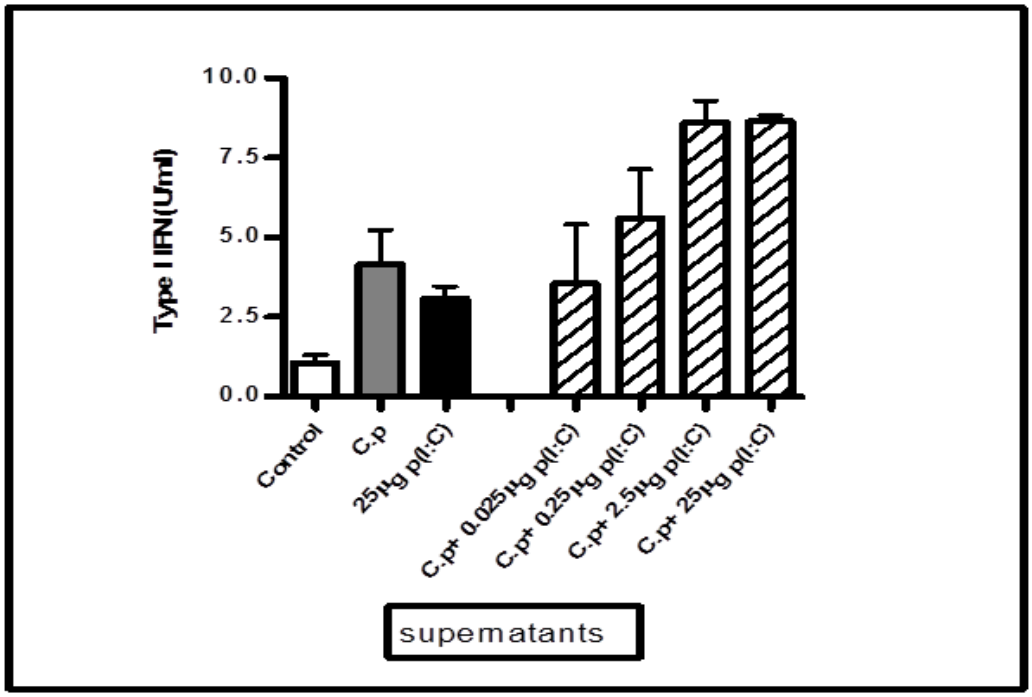

Figure 16. The production of type I IFN in supernatant of cultured CMT-93 cells measured using an antiviral assay. Supernatant from infected CMT-93 cells with or without poly (I:C) treatment showed a significant antiviral activity ($p<0.026$). Also, supernatants from cells cultured with poly (I:C) on its own had significant antiviral activity ($p<0.05$). Samples from uninfected cells had the least antiviral activity.

3.3.1.10 Measurement of IFN-α in supernatants of infected CMT-93 cells

The previous result indicated that supernatants from infected enterocytes can inhibit virus reproduction after being infected with *C. parvum*. This is possibly in part due to the production of type I IFN, but it was important to confirm the production of this cytokine. CMT-93 cells were infected for 24 h after adding one of two doses of *C. parvum* oocysts (2×10^5 and 1×10^6), then culture supernatants were collected and the level of IFN-α was measured by ELISA for IFN-α. Figure 17 shows that significant amounts of IFN-α were measured from supernatants of infected cells in comparison to the supernatants from the uninfected cells ($p<0.01$). These results indicate that *C. parvum* infection induces cultured enterocytes to produce IFN-α.

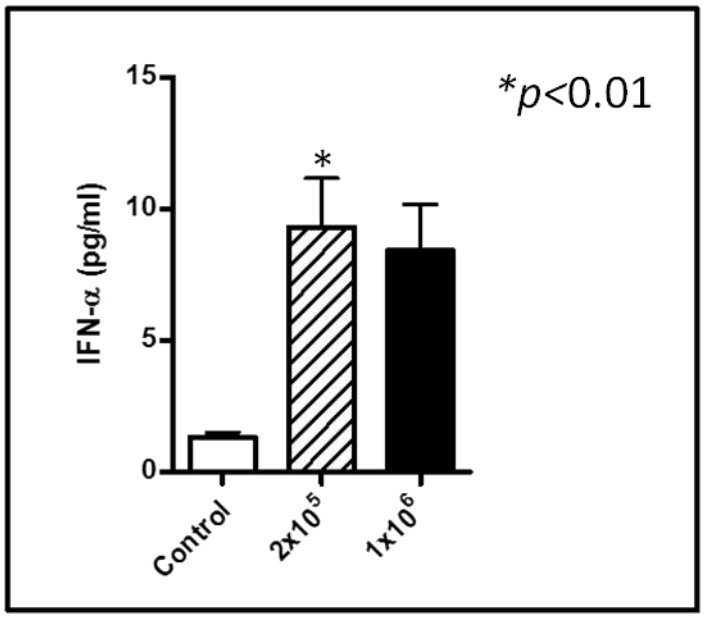

Figure 17.IFN-α expression in supernatants from *C. parvum*-infected CMT-93 cells measured by ELISA. Cells were infected with $2x10^5$ or $1x10^6$ oocysts and supernatants collected after 24h.

3.3.1.11 Type I IFN mRNA expression in infected enterocytes

To further confirm the production of type I IFN by infected cells, measurements were made of IFN-α and IFN-β mRNA by qPCR.

CMT-93 cells in 6 well plates were infected with $1x10^6$ oocysts. After 4 and 8h of infection, RNA from CMT-93 cells was extracted and the levels of IFN-α/β mRNA expression by the infected and uninfected cells were measured by qPCR. Both cytokines were expressed in infected enterocytes. IFN-β was strongly expressed as early as 4 h and continued to be present at 8 h post infection (Fig 18; $p<0.0004$) , while IFN-α was first detected at 8h of infection (Fig 18 ; $p<0.02$).

These results confirm that *C. parvum* infected enterocytes produce type I IFN and this happens early during the course of infection.

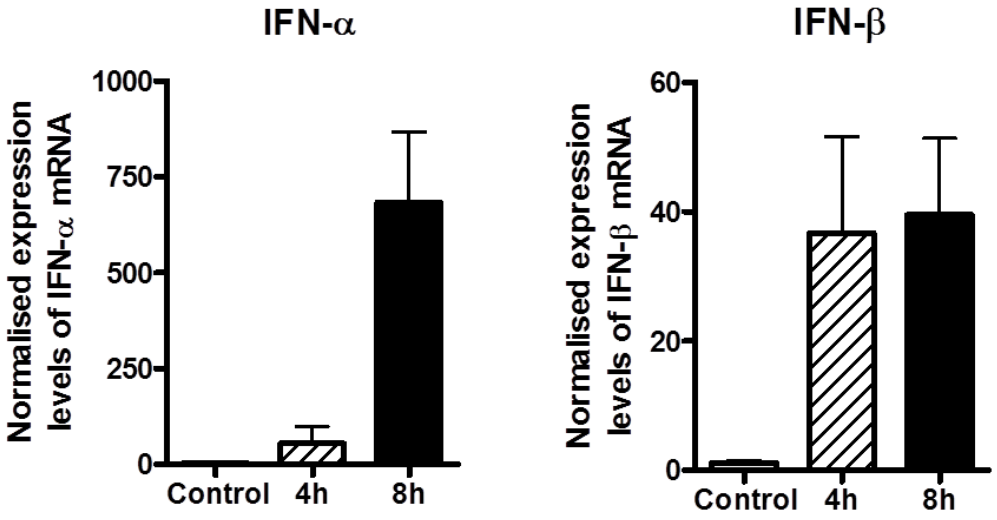

Figure 18. IFN α/β mRNA expression in infected CMT-93 cells 4 and 8h postinfection determined by qPCR. Significant expression of IFN-β was detected at 4h ($p<0.0004$) and persisted at 8h. IFN-α was first measured at 8h post infection ($p<0.02$).

3.3.1.12 The effect of treatment of CMT-93 cells with supernatants from infected CMT-93 cells on the subsequent infection with *C. parvum*

A study was made to determine whether supernatants from infected CMT-93 cells could inhibit *C. parvum* infection *in vitro.* CMT-93 cells were grown on glass coverslips as described previously. The cell monolayers were then incubated for 24 h with supernatant obtained from *C. parvum*-infected CMT cells taken 24 h postinfection or with supernatant from uninfected cells. Then, the cells were infected and the level of parasite development was evaluated 24 h postinfection.

There was a significant reduction in the level of infection in cells which were pre-treated with supernatants of infected cells, while supernatants from uninfected cells had no effect on infection (Fig 19; $p < 0.03$). These results indicate that *C. parvum*-infected CMT-93 cells secrete products that decrease *C. parvum* development *invitro.*

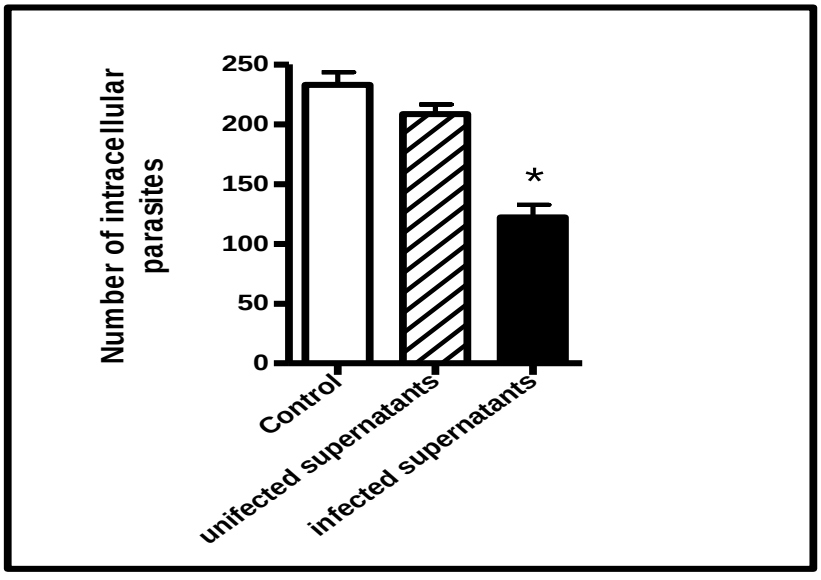

Figure 19.Effect of supernatants from infected and uninfected CMT-93 cells on subsequent *C. parvum* development in other CMT-93 cells. Only supernatants from infected cells inhibited parasite development (*$p < 0.03$).

3.3.1.13 The ability of dendritic cells to express type I IFN

Dendritic cells are important in antigen presentation to T cells as well as in primary inflammation responses. In this experiment the ability of dendritic cells to produce type I IFN after being stimulated by live *C.parvum* oocysts was studied. Bone marrow derived dendritic cells from SCID mice were co-cultured at 37C^{o} with 5×10^{5} liveoocysts (sporozoites excyst at 37C^{o} after about 45 min) or *Salmonella typhimurium* lipopolysaccharide (LPS) as a positive control for 4h and the expression of type I IFN in these cells was analysed by qPCR. Fig 20 A and B show that there was strong expression of both IFN-α and IFN-β mRNA by dendritic cells after 4 hours of exposure to the parasite oocysts or to LPS (p<0.01*; p*<0.001). Similar results were obtained with live sporozoites purified from oocysts and oocyst shell debris by passing through a 5 μm diameter pore filter, and also live sporozoites in the presence of polymyxin B, a potent inhibitor of endotoxin-induced activation (Choudry *et al*., 2009). Purified oocyst surface sterilised with domestic bleach were found not to be contaminated with LPS (data obtained by V. McDonald and D. Korbel). Hence, sporozoites themselves are strong inducers of IFN-α/β.

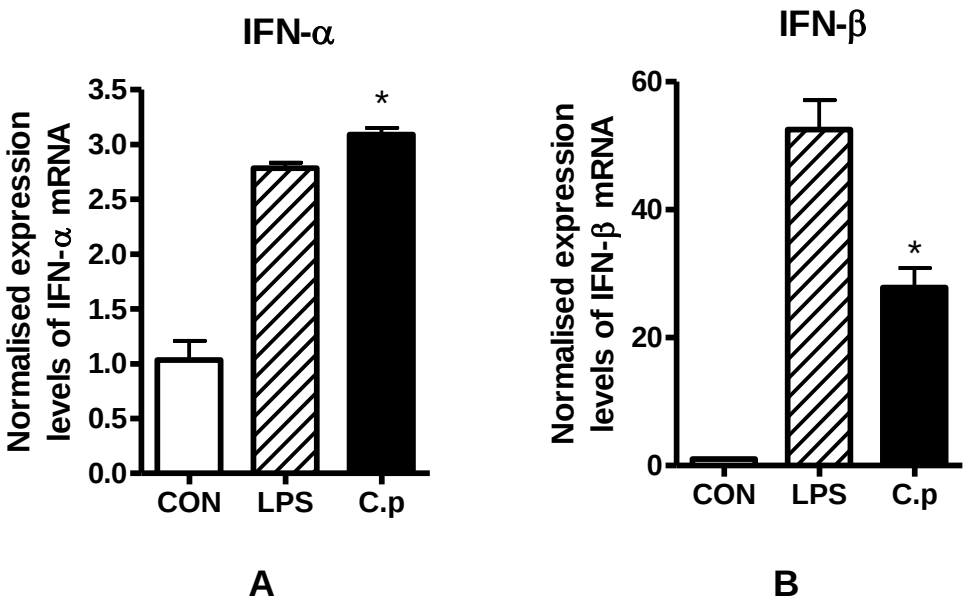

Figure 20 A and B. IFN-α and IFN-β mRNA expression in dendritic cells after 4h stimulation with LPS or *C. parvum* oocysts. Significant expression of IFN-α (*p*<0.01*)* and IFN-β *(p*<0.001*)* mRNA was measured following exposure to C. *parvum* oocysts.

3.3.2 *In vivo* infection studies

The above *in vitro* results indicated a possible protective role for type I IFN against *C. parvum* infection. Hence it was necessary to further investigate the role for Type I IFN in immunity to *C. parvum* in mice.

3.3.2.1 *C. parvum* infection of neonatal BALB/C mice

An initial experiment was designed to study the pattern of infection in neonatal BALB/c mice (adult wild-type mice are resistant to infection) and to indicate the day(s) of maximal infection.

Seven-day old baby mice were infected with *C. parvum* by oral gavage. Groups of mice were sacrificed at different days postinfection and the infection was measured by counting oocysts microscopically in colonic faecal smears stained by the Ziehl-Neelsen acid fast method (Fig 21).

Fig 22 shows that there were no parasites detected until 4 days postinfection. The largest numbers of oocysts were produced on days 5-7 and there was a downward trend in numbers excreted after day 5. By day 10 only a few oocysts were seen.

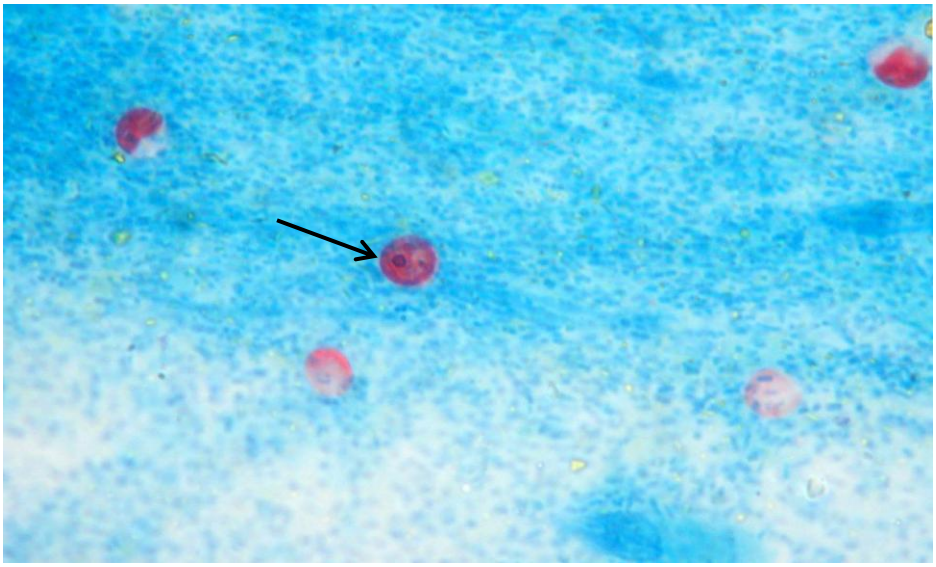

Figure 21. Acid fast-stained colonic stool smears indicating *C. parvum* oocysts (arrowed).

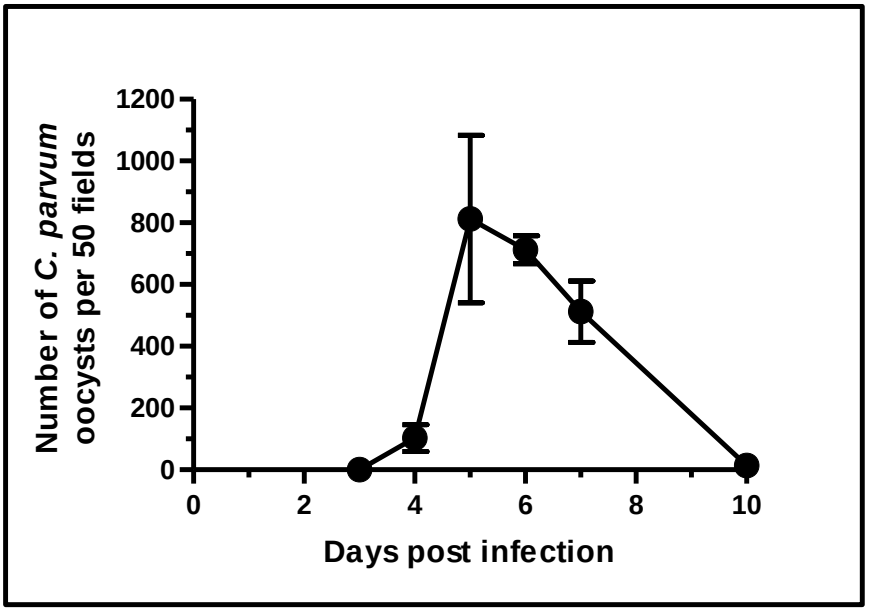

Figure 22. Pattern of oocysts production by neonatal BALB/C mice infected with *C. parvum.* High levels of infection were observed 5-7 days postinfection. At day 10 only a few oocysts were seen.

3.3.2.2 *C. parvum* infection of neonatal SCID mice

As in the previous experiment with BALB/c mice infection was followed daily in neonatal SCID mice (that lack B and T cells) by counting the number of parasites in colonic stool smears. Figure 23 indicates that all mice were significantly infected by day 4 of infection and continued to shed parasites in the following days until day 7. The infection level dropped significantly at day 8 postinfection.

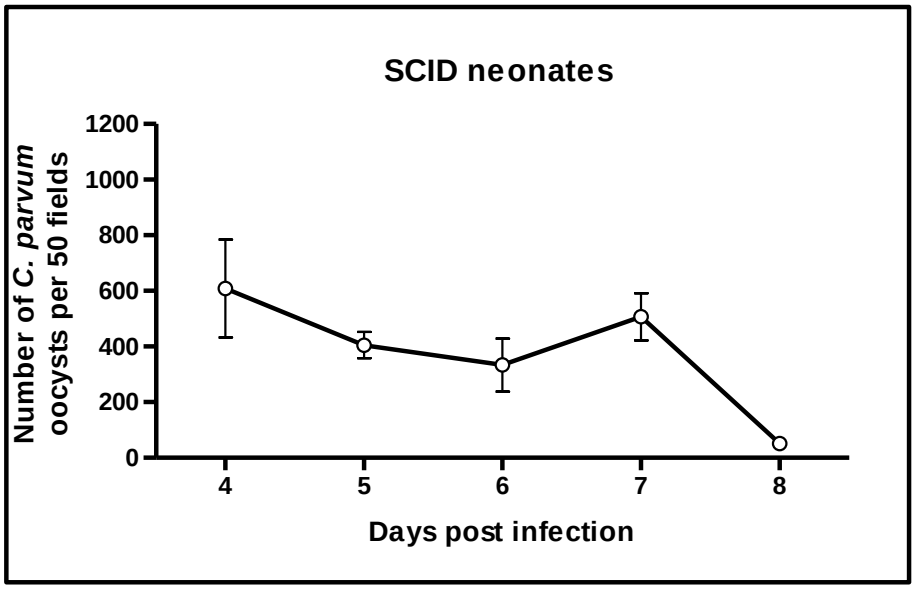

Figure 23. Pattern of *C. parvum* infection in neonatal SCID mice
Significant levels of infection were observed between day 4 and 7 postinfection with a rapid drop of oocyst shedding occurring by day 8 postinfection.

3.3.2.3 The role of type I IFN in immunity to *C. parvum* in neonatal immunocompetent BALB/c mice

Seven day old BALB/c mice were treated with 100 µl anti-mouse type I IFN neutralizing serum or control serum and infected with *C. parvum* oocysts. At day 6 postinfection which is during the peak period of infection, the animals were sacrificed and the number of oocysts in colonic contents smears was counted microscopically. It was found that the level of infection in anti-type I IFN antibody-treated mice was significantly higher than in the control group (Fig 24; p=0.001*)*.These results are the first to signify a role for type I IFN in immunity to *C. parvum invivo*.

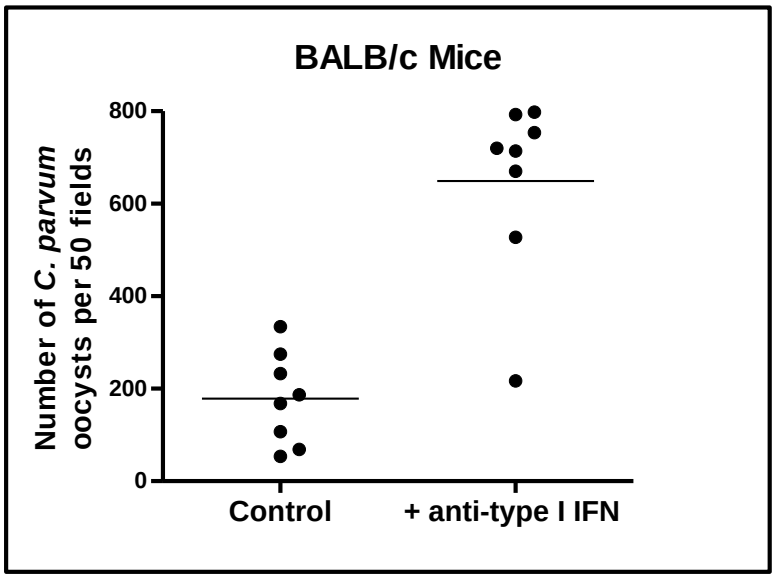

Figure 24.Effect of anti-type I IFN antibody on *C. parvum* infection in neonatal BALB/C mice. Levels of *C. parvum* infection were measured in colonic stool smears of control mice and anti-type I antibody-treated mice 6 days postinfection. Higher numbers of oocysts were observed in samples collected from the anti-type I IFN serum-treated mice in comparison to the mice treated with control serum (p=0.001).

3.3.2.4 The role of type I IFN in immunity to *C. parvum* in neonatal immunocompromised SCID mice

The previous results indicated a protective role for type I IFN in immunity to *C.parvum* in immunocompetent neonatal mice. A similar study was performed to determine if type I IFN was also important for control of infection in immunocompromised SCID mice.

Seven-day old SCID mice were injected with 100 µl of anti-type I IFN serum or control serum and infected with *C. parvum*. The infection level was measured at day 6 postinfection when colonic stool samples were collected and parasite oocysts were counted in acid-fast stained smears.

Similar to the results seen with BALB/c mice, the number of oocysts in stool samples from SCID mice treated with anti-type I IFN was higher than in samples obtained from control mice (Fig 25; $p<.0.001$). These results confirm the role of type I IFN in innate immunity to *C. parvum* infection.

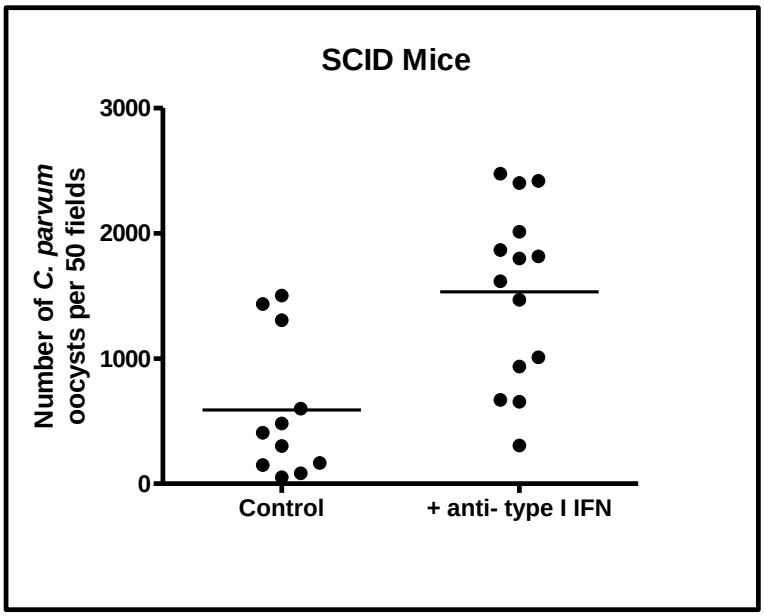

Figure 25.Role of type I IFN in the control of *C. parvum* infection in neonatal SCID mice. Neonatal SCID mice were treated with anti-type I IFN serum or control serum and the level of infection was assessed at day 6 postinfection. Results show a significant rise in parasite shedding in stool samples obtained from anti-type I IFN- treated mice in comparison to the mice treated with the control serum ($p<.0.001$).

3.3.2.5 The role of type I IFN in immunity to *C. parvum* in neonatal SCID mice early during infection

Previous results showed that type I IFN had a protective effect against infection when studied 6 days post-infection and *in vitro* infected cells produced type I IFN after 4-8 h of infection. It was important to know, therefore, if type I IFN-dependent immunity can be shown early in infection of baby mice.

After treatment with anti-type I IFN serum, 7 day-old mice were infected by inoculation with a larger than normal number of oocysts ($1X10^5$) and the mice were sacrificed 48h post-infection. Early stages of infection in mice were measured semi-quantitatively by counting parasites developing in intestinal villi, as oocysts were unlikely to be produced by 48h postinfection. At 48 h there was a significant increase in the number of infected cells in sections from anti-type I IFN antibody treated mice when compared to the control mice (Fig 26; $p<0.01$). These results indicate therefore that type I IFN is a key element of the early innate immune response to *C. parvum* infection.

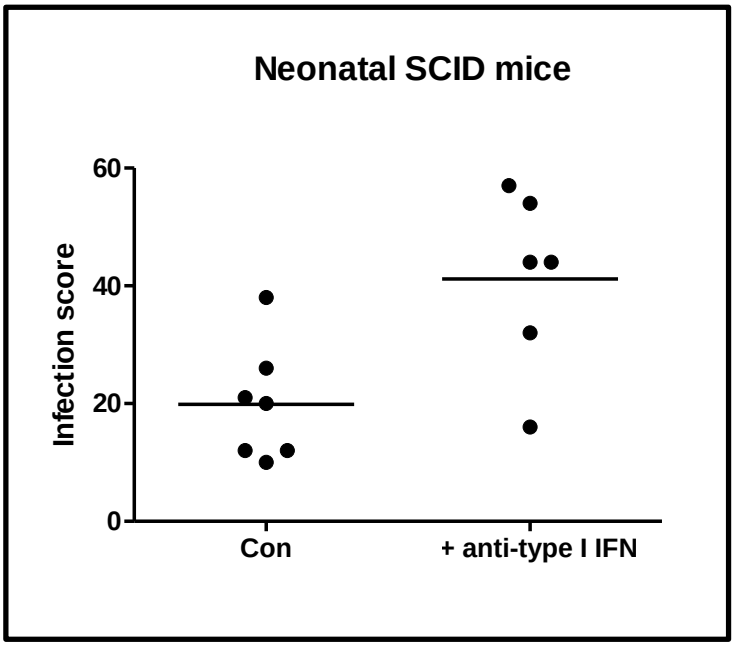

Figure 26.The role of anti-type I IFN in the early infection of neonatal SCID mice. Neonatal mice were treated with anti-type-I IFN serum or control serum, infected with a high number of oocysts and the level of infection in the intestine was measured 48h postinfection by microscopic examination of haematoxylin/eosin-stained sections of ileum. A significant increase (p<0.01) in the level of infection was observed in intestinal samples obtained from anti-type I IFN serum-treated mice in comparison to the mice treated with the control serum.

3.3.2.6 The expression of type I IFN in the intestine of SCID mice during early infection with *C. parvum*

It was evident from previous results that type I IFN is an important element in early innate immunity to *C. parvum* in neonatal mice. It was then necessary to confirm the expression of type I IFN in the intestine of infected mice. SCID mice were infected with *C. parvum* oocysts for 24 h or 48 h. The animals were then sacrificed and the expression of type I IFN mRNA in the ileum was measured by qPCR in samples.

As shown in Fig 27, both cytokines were found in ileal tissue as early as 24 h postinfection in comparison to tissues from uninfected mice ($p<0.04$) ,($p<0.0002$) and while the expression of IFN-α mRNA in infected mice was still observed after 48h ($p<0.037$), IFN-β was not detected at that time.

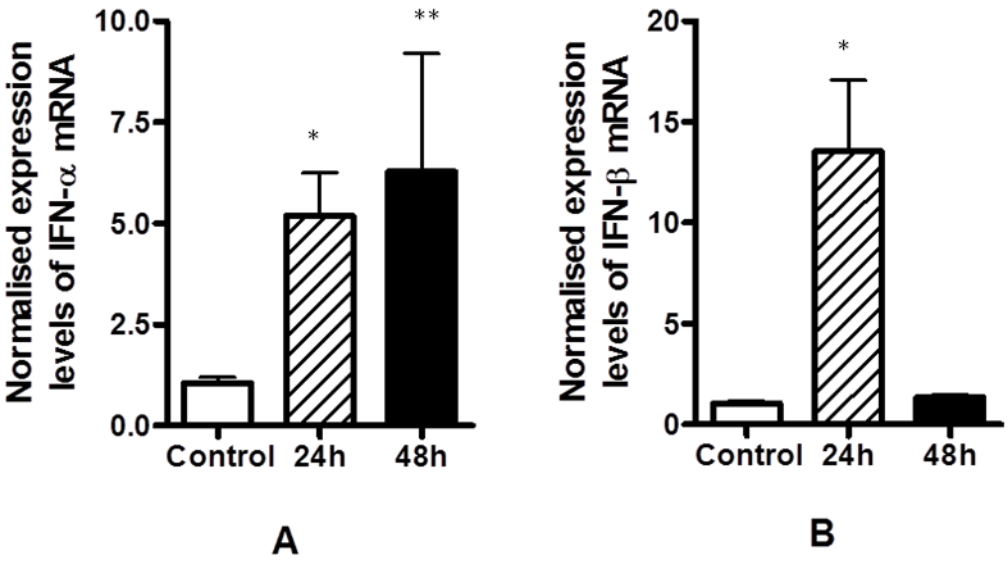

Figure 27 A and B. Expression of IFN-α and IFN-β mRNA in ileal tissue 24 h and 48 h postinfection determined by qPCR. IFN-α was detected at 24h and 48h postinfection (*$p<0.04$; **$p<0.037$), while IFN- β was detected at 24 h after infection (*$p<0.0002$).

3.3.2.7 The expression of IFN-γ in intestinal samples of infected SCID mice with or without treatment with anti- IFN type I serum

IFN-γ is a key cytokine in innate immunity to *C. parvum* in SCID mice as shown previously by this group (McDonald *et al.,* 2000). To investigate whether the immunity acquired by type I IFN is related to the production of IFN-γ, the intestinal expression of IFN-γ mRNA was measured by qPCR 48 h postinfection from neonatal mice pre-treated with anti-IFN-α/β or control serum. IFN-γ mRNA was not detected in uninfected mice but was significantly expressed in infected mice (p<0.027). There was stronger expression of IFN-γ mRNA in anti-type I IFN serum-treated mice in comparison to infected controls. However this was not quite significant (Fig 28; p=0.0511).These results indicate that *in vivo* the type I IFN inhibitory function may not be dependent on the production of IFN-γ.

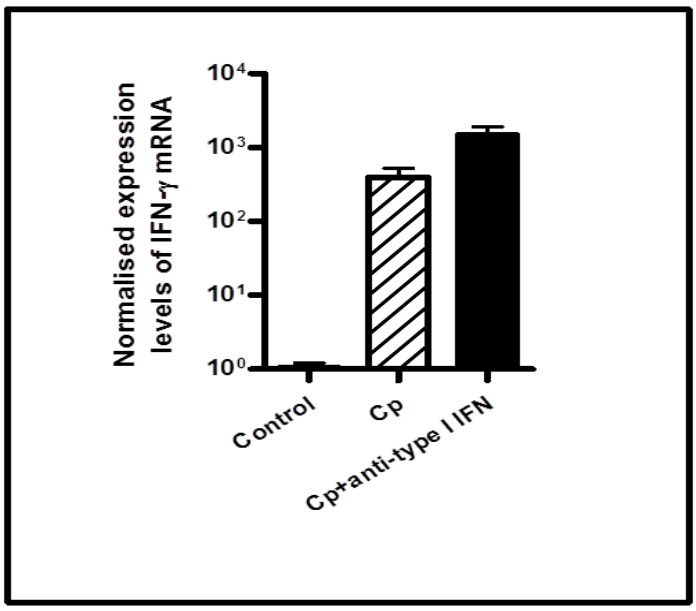

Figure 28.The effect of the treatment with anti-type I IFN antibody on the expression of IFN-γ mRNA, in ileal tissue. Neonatal SCID mice were treated with anti-type I IFN or control serum and IFN-γ expression determined by qPCR 48 h postinfection. No significant difference in the level of IFN-γ expression was detected between the two groups (p=0.0511).

3.3.2.8 The effect of TLR3 agonist (poly (I: C)) on *C. parvum* infection of SCID mice

The previous *in vivo* results have demonstrated that enterocytes are capable of producing type I IFN after infection with *C. parvum* and they also indicated a role of type I IFN in limiting the infection *in vivo* as shown in experiments with treatment of mice with anti-type IFN I antibody. Also *in vitro* studies showed a role of the TLR3 agonist (poly (I: C)) in limitation of *C. parvum* development possibly in part via the production of type I IFNs.

In this study, the immunomodulatory effect of poly (I: C) on the infection with *C. parvum in vivo* was studied. Seven–day old SCID mice were infected with *C. parvum* oocysts as described previously and were treated intraperitoneally with three doses of 100 µg of Poly (I: C) at days -1, 0, and +1 of infection or with PBS as control. The dose of poly (I:C) used in this experiment was similar to the dose used to study the effect of poly (I:C) on infection of SCID mice with *Candida albicans* (Jensen *et al.,* 1992).

Five days postinfection mice were sacrificed and the level of infection was measured microscopically in acid fast stained smears of colonic samples. A significant reduction of infection was observed in the poly (I: C) treated mice in comparison to the control mice (Fig 29; p= 0.01).

These results indicate that the exogenous treatment of SCID mice with the IFN inducer poly (I: C) decreases *C. parvum* reproduction.

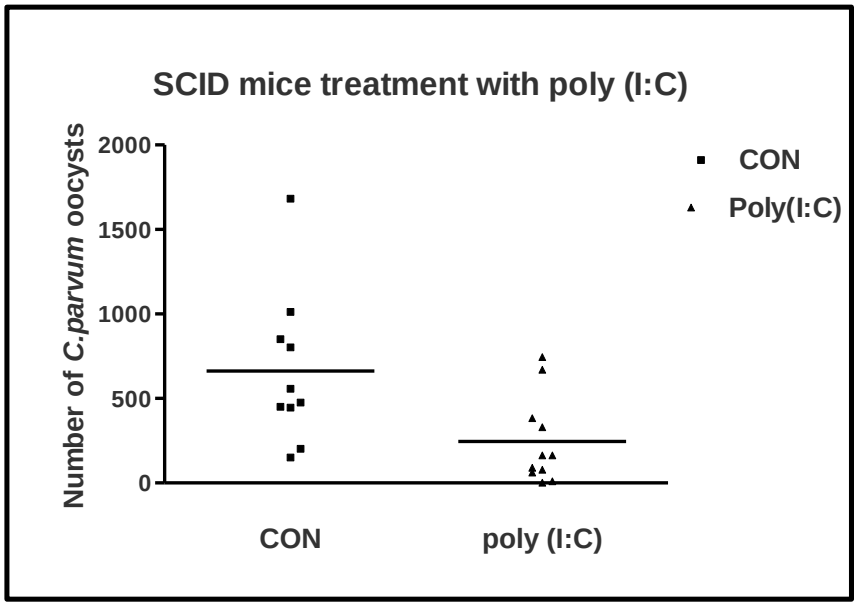

Figure 29. The effect of Poly (I:C) treatment on *C. parvum* infection of SCID mice. Neonatal SCID mice were infected with *C. parvum* and were treated i.p. with 300 µg of poly (I:C) or control. A significant reduction in the number of parasites (*p=0.*01) was detected in the poly (I:C) treated mice in comparison to the control group when measured at day 5 post infection.

The previous results indicated that the exogenous treatment of SCID mice with type I IFN inducer poly (I: C) decreases *C.parvum* development. So it was essential to investigate the effect of treatment of neonatal mice with exogenous IFNs on the infection.

Mice were infected at 7 days of age and treated subcutaneously with $1x10^4$ U of IFN-α 4 in PBS or PBS on days -1, 0, and +1. Mice were sacrificed on day 5 postinfection and colonic stool smears examined microscopically as in previous experiments. No difference in the level of infection was observed between the IFN-treated mice and the control (results are not shown).

3.4 DISCUSSION

Type I IFNs are a multi-gene family of cytokines with strong antiviral activity acting alone or with other cytokines to clear infections (Takaoka and Yani, 2006). They also represent potent modulators of different immune responses (Chelbi-Alix and Wietzerbin, 2007). Apart from the antiviral effects, type I IFNs were found to play roles in immunity to non-viral microbial organisms including bacterial, protozoal, and fungal infections (Bogens *et al.,* 2004). The effects were either inhibitory or pathogen aggravating, depending on the pathogen and the mechanism(s) of control (Bodgen *et al*., 2004).

In *Cryptosporidium* infection, different cytokines have been found to play roles in the innate immune response and IFN-γ is a key cytokine. The role of type I IFNs in immunity to *Cryptosporidium* has not previously been studied. In the present study the role of type I IFNs in resistance to *C. parvum* infection was investigated.

Using an *in vitro* model of infection recruiting the human enterocyte cell line Caco-2 and murine CMT-93 cells the effect of type I IFNs on *C. parvum* infection was studied.

Treatment of Caco-2 cells with IFN-α inhibited parasite development in a dose-dependent manner and a concentration of 0.1 U/ml IFN-α was sufficient to inhibit parasite reproduction. Similar inhibitory effects on *C. parvum* development in HT-29 cells were previously detected with IFN-γ and TNF-α (Pollok *et al.,* 2001). In Caco-2 cell infection, a dose of 1 U/ml of IFN-γ was found to inhibit parasite development by about 60% and the inhibitory effect increased with higher doses of the cytokine. The effect of lower concentrations of the cytokine was not tested (Pollok *et al.,* 2001). Interestingly, with rotavirus infection, pre-treatment of Caco-2 and HT-29 cells with IFN-γ but not IFN-α induced resistance to the virus (Bass, 1997).

In this study, both IFN-α and IFN-β were found to inhibit *C. parvum* development in cultured CMT-93 cells suggesting that the anti-parasitic activity of type IFNs may be general and not specific to one host type.

Different antimicrobial killing mechanisms have been associated with type I and type II IFN activity against intracellular microbial pathogens. Therefore it was important to determine possible anti-parasitic mechanisms of type I IFN in resistance to *C. parvum.*

Previous *in vitro* studies indicated that *Cryptosporidium* needs about 2-3 h to invade the epithelial cells. Both IFN-α and IFN-γ were found to inhibit *Shigella* invasion of epithelial cells, although IFN-γ was found to be more potent (Niesel *et al.*, 1986) Therefore, it was important to investigate the effect of IFN-α on the early stage of infection by *C. parvum*. A significant reduction (about 50%) of parasite numbers was observed in IFN-α-treated Caco-2 cells 3 h postinfection. Hence prevention of host cell invasion may be an important mechanism of action of type I IFNs against *C. parvum.* A similar (but not major) mechanism of action was previously proposed for the action of IFN-γ against *C. parvum* infection of cultured enterocytes (Pollok *et al*., 2001). In *Shigella flexneri* infection, IFN-α inhibited a Src-dependent signaling cascade triggered by *Shigella* that leads to the reorganization of the host cell cytoskeleton and bacterial entry into the cell (Dumenil *et al.,*1998). Another *in vitro* study with *C. parvum* infection had shown that inhibition of cytoskeletal rearrangement in host cell cells with a drug may prevent invasion of the host cell by the parasite (Forney *et al.,* 1999). However, in the current project the effect of type I IFNs on key proteins known to be involved in cytoskeletal organisation was not investigated and it would be important to undertake further studies in that area in future.

The results obtained with IFN-γ indicated that the cytokine had a greater inhibitory effect on parasite development than on invasion (Pollok *et al.,* 2001). However, in

this study IFN-α did not appear to have any additional inhibitory effects after 3 h postinfection. This was shown by comparing the relative level of infection at 3, 8 and 24 h postinfection in cytokine-treated versus control cells. In addition, at 8 h after infection counts were made of parasite developmental stages present in cytokine and control cells and the results indicated that IFN-α did not appear to affect the rate of maturation of intracellular parasites.

Pollok *et al.* also found that IFN-γ inhibited *C. parvum* development by the deprivation of cellular $Fe^{2+,}$ which is an important component for microbial pathogen growth (Pollok *et al.,* 2001). In the present study this mechanism did not appear to be involved since the supplementation of the cell monolayer with increased Fe^{+2} (by addition of Fe_2SO_4 as described by Pollok *et al*.) did not reduce the inhibitory effect of IFN-α.

Depletion of cellular L-tryptophan by IFN-γ has been shown previously to help the host cell to resist the development of different pathogens. Indoleamine 2,3 dioxygenase (IDO) enzyme can catabolise tryptophan required for microbial growth and the expression of IDO gene is induced only in response to inflammatory stimuli such as type I and II IFNs (King and Thomas, 2007). It was previously shown that IFN-γ inhibited *T. gondii* development in fibroblasts by tryptophan starvation which could be reversed by addition of exogenous tryptophan (Ceravol *et al.,* 1999). In this laboratory tryptophan was also found to prevent IFN-γ-induced killing of the intestinal microsporidian *Encephalitozoon intestinalis* in CMT-93 cells and it was also shown that the killing was dependent on IDO expression and activity (Choudhry *et al*., 2009). However, this treatment did not affect either the anti-parasitic effect of IFN-γ against *C. parvum* infection (Pollok *et al.,* 2001) or of IFN-α activity in Caco-2 cells in the present study. Although it is important to mention that the medium used in the current study is full DMEM which contains both tryptophan and iron. Therefore this leaves the possibility that each of these could play a role if they were present in limiting conditions. Particularly for the experiment investigating the effect of

tryptophan as the concentration of tryptophan u added in an attempt to rescue the effects of IFN was much lower than the one present in standard DMEM. A tryptophan free medium or higher concentration of tryptophan could overcome this problem and should be considered in future work.

During *C. parvum* as well as other protozoal infections, epithelial cells produce nitric oxide via the upregulation of iNOS in the presence of IFN-γ and other proinflammatory cytokines (Kolios *et al.,* 1998), but NOS inhibition by N^{G}-NMA was not found to affect the action of IFN-γ against *C. parvum* infection of the enterocytes, indicating IFN-did not kill the parasite via NO production (Pollok *et al.,* 2001). In a previous study by Dienfenbach *et al.* with *Leishmania major* it was found that NO was produced significantly when macrophages were simultaneously exposed to *L. major* promastigotes plus IFN-α/β *in vitro*. It was also observed in mice infected with *L. major* that the early production of iNOS was dependent on IFN-α/β. These results indicated that these cytokines together with the parasites provided signals for the induction of iNOS *in vivo* (Dienfenbach *et al.,* 1998). Another study by Mattner *et al.* confirmed a protective role for IFN-β against cutaneous and visceral *L. major.* This was associated with up-regulating IFN-γ, increasing tyrosine phosphorylation of Stat1 and Stat4 and increasing expression of iNOS (Mattner *et al.,* 2004). In bacterial infections, type I IFNs also played a protective role via the production of IFN-γ and NO. In *Chlamydia pneumonia,* type I IFNs enhanced the production of IFN-γ and NO in cultured macrophages that helped in resisting infection (Rothfuchs *et al*. 2001). In a study of the infection with group B streptococci (GBS), type I IFN signalling was found to be essential in immunity against infection as IFN-α/β$R^{-/-}$ mice were more susceptible to infection than the wild type mice. Also it was observed in the same study that the production of IFN-γ, NO and TNF-α measured by ELISA was significantly reduced in

supernatants obtained from macrophages obtained from IFN-α/βR$^{-/-}$ after stimulation with GBS for 24 h. (Mancuso *et al*., 2007).

It was important therefore in the current study to elucidate the role of NOS as the mechanism of action of type I IFNs. No effect was detected with the NOS inhibitor N^{G}-NMA on the inhibitory action of IFN-α. This indicates that NOS had no role in parasite killing mediated by IFN-α. However, arginine may play a protective role against cryptosporidial infection by the alternative activation of macrophages in which arginase activity is induced by IL-4 (Castro *et al.,* 2012) and a study from this laboratory has demonstrated that IL-4 is important in innate immunity against *C. parvum* (McDonald *et al.,* 2004). Indeed it has been shown that arginine is an essential amino acid for development of *C. parvum* (Castro *et al.,* 2012). Also, a recent study from our groupobserved that macrophages had a key role in innate immunity as their depletion in mice strongly exacerbated infection (Choudhry *et al.,* 2012).

After establishing that type I IFN plays a protective role against *C. parvum* infection through inhibiting parasite invasion of the epithelial cells, it was of interest to investigate if the epithelial cells were capable of producing type I IFN in response to *C*. *parvum* infection. The TLR3 agonist poly (I:C) is a synthetic dsRNA known to be a potent inducer of type I IFN (Field *et al.,*1967). Studies by others indicated treatment of epithelial cells with poly (I:C) enhanced the antiviral activity of the cells (Schaefer *et al.,* 2005). Also, *in vivo* studies indicated a protective role for poly (I:C) against infections with other microbial pathogens (Herman and Baron, 1970).

It was shown in the current study that treatment of CMT-93 cells with poly (I:C) induced protection against *C. parvum* infection and this was associated with the induction of type I IFN. There was a significant reduction in parasite numbers in poly (I:C)-treated cells. To examine the effect of poly (I:C) or *C. parvum* infection on enterocyte expression of type I IFN, the production of type I IFN was measured indirectly by employing a bioassay of antiviral activity. Poly (I:C) or *C. parvum*

alonestimulated CMT-93 cells to produce type I IFN and in the presence of both infection and poly (I:C) treatment, even higher levels of type I IFN were detected. These results indicate that enterocytes are capable of producing type I IFN after infection with *C. parvum* or treatment with poly (I:C).

Further experiments were conducted in this project to study the capability of epithelial cells to express type I IFN in response to *C. parvum* infection. IFN-β mRNA was expressed by CMT-93 cells within 4 h of being infected as measured by qPCR and both IFN-α and IFN-β were detected at 8 h. The early expression of IFN-β correlates with the overall picture obtained by previous studies indicating that upon infection, high constitutive levels of IRF-3 and low levels of IRF-7 lead preferentially to IFN-β transcription; this is then followed by a secondary phase mediated by IFN-β that creates a high level of IRF-7 expression that favours transcription of most IFN-α genes (Ford and Thanos, 2010). A significant amount of IFN-α was detected in supernatants derived from *C. parvum* infected CMT-93 cells after 24 h of infection when measured by ELISA, confirming the expression of this cytokine as a result of *C. parvum* infection.

In further experiments in the present study, a significant inhibition of parasite development was observed in CMT-93 cells pre-treated with culture supernatants originating from *C. parvum*-infected CMT-93 cells taken 24 h postinfection while supernatants from uninfected monolayers had no effect on *C. parvum* development. This suggests that infected enterocytes secrete products (including type I IFN) that help to limit *C. parvum* infection.

The above results indicate that cultured enterocytes express type I IFNs in response to *C. parvum* infection and that enterocytes secrete products that help inhibit the infection, including type I IFNs. It was not established, however, that type IFNs in supernatant from infected enterocytes played a major role in inhibiting *C. parvum* infection. This could have been examined by adding anti-type I IFNs neutralising

antibody to supernatants from the infected cells when added to fresh infected cells and determining whether the supernatant was still capable of inhibiting *C. parvum* development. Unfortunately there was insufficient time to carry out this experiment. It is possible that production of antimicrobial peptides (see below) or other cytokines by infected cells may in addition to type I IFNs help to protect the epithelium.

The mechanism(s) by which type I IFN expression is induced in infected enterocytes is not known, TLRs are well known inducers of type I IFNs expression (Noppert *et al*., 2007) and a protective role for TLRs in immunity to *C. parvum* infection has been previously described (Costa *et al.,*2011). Chen *et al.* observed that *C. parvum* infection of cultured cholangiocytes (bile duct epithelial cells which become infected in immunocompromised hosts) induced the recruitment of TLR2 and TLR4 at the site of parasite attachment/invasion and those receptors were involved in initiating the inflammatory responses and β-defensin-2 production of infected cells (Chen *et al*., 2005). Most TLRs initiate signalling by recruiting MyD88 and MyD88 deficient mice were more susceptible to *C. parvum* infection than wild type mice (Rogers *et al.,* 2005). Also, treatment of neonatal mice with the TLR9 ligand (unmethylated CpG oligonucleotide) stimulated strong resistance to *C. parvum* infection (Barrier *et al.,* 2006). Therefore, it is possible that TLRs are involved in inducing type I IFN expression as a result of *C. parvum* infection.

The role of dendritic cells in establishing early innate immune and adaptive responses to various infections is well documented (Moretta *et al.,* 2002). Dendritic cells are also known as an important source of type I IFNs (Colonna *et al*., 2004). Significant IFN-β and IFN-α mRNA expression by dendritic cells was observed after 4 h of exposure to excysting *C. parvum* sporozoites. Similar results were obtained with live sporozoites purified by passage through a filter with 5μm diameter pores. An ultrastructural study of *Cryptosporidium* infection of guinea pigs showed parasite invasive stages engulfed by mononuclear cells adjacent to the epithelium of Peyer's

patches (Marcial and Madara, 1986) where a large population of dendritic cells becomes located following infection (Iwasaki and Kelsall, 2001). An *in vitro* study by Auray *et al.* indicated that *C. parvum* infected epithelial cells grown on an insert membrane secreted chemokines that attracted dendritic cells in the well below into the membrane (Auray *et al.,* 2007). Taken together these observations suggest that dendritic cells have an important role in association with type I IFNs in immunity early during infection with *C. parvum.*

A previous study with *Schistosoma mansoni* infection, showed that the helminth eggs stimulated dendritic cells for the expression of IFN-β and various IFN-inducible genes and this was dependent on IFNα/β receptor signaling (Trottein *et al.,* 2004). In *Salmonella typhimurium* infectionboth MyD88 and IFN-α/β were found to be important for dendritic cell activation of T cells (Tam *et al.,* 2008). In a recent study of the role of type I IFNs in immunity to *P. falciparum* it was shown that PBMCs stimulated with schizont-infected RBCs induced IFN-α at the protein level and IFN-β mRNA, but the cellar source of IFN was not established (Sharma *et al.,* 2011).

The above results indicated that *C. parvum* inducedenterocytes and dendritic cells to produce type I IFNs. In addition IFN-α/β inhibited parasite development in enterocytes. Hence it was essential to investigate the possible role of type I IFNs in immunity to *C. parvum in vivo*. This was studied using neonatal BALB/c wild type and immunocompromised SCID mice.

Many studies conducted previously investigated the role of type I IFNs in immunity to different microbial pathogens. IFNAR$^{-/-}$ knockout mice have been a valuable tool used by different investigators to investigate the role of type I IFNs in immunity. For example, in cutaneous leishmaniasis, IFNAR1$^{-/-}$ mice developed smaller lesions when infected with the South American parasite *Leishmania amazonensis* in comparison to wild type mice, indicating a role for type I IFN in promoting disease (Xin *et al.,* 2010). In contrast, in infection with group B streptococci, wild type mice

survived infection whereas mortality was observed in IFNAR1$^{-/-}$ and IFN-β $^{-/-}$ mice. This confirmed that type I IFN signaling was important in resistance against this infection (Mancuso *et al*., 2007).

In this laboratory the 129SV/Ev wild type and IFNAR1$^{-/-}$ neonatal mice were originally used to study the role of type I IFNs in *C. parvum* infection. Both wild type and mutant mice had very low levels of infection, however, suggesting that 129SV mice were naturally resistant to infection with *C. parvum* (for unknown reasons) (V. McDonald, unpublished data). Hence the 129S strain is not suitable for studying *C. parvum* infection.

The use of anti-IFN-α/β antibodies to study the role of type I IFNs in virus infections was first described in 1976. Gresser *et al.* found that treatment of mice with anti-IFN-α/β antibodies developed in sheep enhanced infection with encephalomyocarditis (EMC) virus (Gresser *et al.,* 1976). Sheep anti-mouse IFN-α/β antibodies in serum produced and titrated by our collaborator Dr M.G. Tovey (Institut Andre Lwoff) commonly used by many groups, were used in the present study to investigate the role of type I IFNs in immunity to *C. parvum* in mice. An important finding was that the treatment of neonatal BALB/c and SCID mice with anti-IFN-α/β antibodies prior to infection caused a significant increase in oocyst shedding around the peak of infection in comparison to the mice that received control antibodies. These results indicate a protective role for type I IFNs, at least in innate immunity, against *C. parvum in vivo*.

It was also found that at 48 h postinfection, pretreatment of SCID mice with anti-IFN-α/β resulted in increased parasite numbers in intestinal villi compared with the controls. This confirms that type I IFNs mediate inhibition of parasite development early during infection.

In view of these findings, it was important to measure the expression of type I IFN in the intestine of infected mice. At 24 h and 48 h of infection ileal samples from SCID mice were tested for the expression of IFN-α and IFN-β mRNA by qPCR. Both IFN-

α and IFN-β expression was detected at 24 h postinfection, but only IFN-α response was maintained at 48 h.

The termination of IFN-β expression after 24 h postinfection is not unexpected and can be explained at the transcription level. Transcription of IFN-β requires the formation of a large multi-protein complex named the enhanceosome that includes NF-κB, IRF-3/7 and ATF-2/c-Jun (Hiscott, 2007). At the peak of transcription, an architectural protein of the complex HMGA1 that stabilises the enhanceosome and facilitates DNA binding is acetylated by another component, the transcriptional co-activator CBP that is associated with the RNA polymerase II complex bound to the enhanceosome. Increasing acetylation destabilises the enhanceosome leading to IFN-β transcription being turned off (Ford and Thanos, 2010).

In mice infected with *Trypanosoma cruzi,* a transient peak of IFN-α was detected at 24 h of infection (Kierszenbaum and Sonnenfeld, 1982). However, no difference in susceptibility to infection or the production of IFN-γ was observed between $IFNAR^{-/-}$ mice and wild type mice.

IFN-γ is a key cytokine in the control of *C. parvum* infection and it is known that type I IFNs can activate NK cells to produce IFN-γ in response to different microbial pathogens (Korbel *et al.,* 2004). In infection with *Toxoplasma gondii* treatment with recombinant IFN-β enhanced protection against infection and this was dependent on IFN-γ production (Orellana *et al.,* 1991).

It was relevant, therefore, to study if the protective function of type I IFNs in *C. parvum* development was related to IFN-γ expression. Treatment of neonatal SCID mice with anti-type I IFN-α/β caused an exacerbation of infection but this was not associated with reduced intestinal IFN-γ expression when measured at day 2 postinfection. In fact, there was higher IFN-γ expression in the anti-IFN-α/β-treated mice, although this was not statistically significant. It is possible that the higher expression could relate to higher level of infection in anti-IFN-α/β-treated mice.

Other possible mechanisms for the protective action of type I IFNs action have been discussed in this chapter but have not been investigated during the present study.

It was shown in this study that poly (I:C) treatment of cultured enterocytes stimulated the cells to produce type I IFNs and this effect was increased when the cells were infected with *C. parvum*. In previous *in vivo* studies of other microbial pathogens it was found that poly (I:C) treatment enhanced protection against infection. For example, treatment of mice with poly (I:C) prior to infection with *Leishmania donovani* caused a significant decrease in parasite reproduction along with a remarkable rise in IFN-α/β levels in the serum (Herman and Baron, 1970). Therefore, it was of interest to investigate the effect of exogenous poly (I:C) on *C. parvum* infectionof SCID mice. A significant decrease of parasite numbers was observed in colonic stool samples obtained from poly (I:C)-treated mice in comparison to the control group after 5 days of infection. This indicated that treatment with poly (I:C) enhanced immunity against *Cryptosporidium* infection, however the effect on type I IFN production of poly (I:C) stimulation was not measured in this *in vivo* infection model.

Confirming these findings, a recent study by Lamande *et al.* at The National institute for Agronomical Research, Tours, France, has indicated that a single dose of poly (I:C) induced protection against *C. parvum* infection in wild type neonatal mice (Dr Sonia Lamande, personal communication and data presented at the Mucosal Immunology Conference, Paris, July 2011).

On the other hand, poly (I:C) treatment caused an exacerbation of infection in wild type mice but not $IFNAR^{-/-}$ mice infected with *L. monocytogenes* or with *M. tuberculosis* (O'Connell *et al.,* 2004; Antonelli *et al.,* 2010). Also, treatment of SCID mice with poly (I:C) increased susceptibility to systemic candidiasis (Jenesen *et al.,* 1992). Although the dose and method of injection used in the current study was similar to that described by Jenesen *et al.* anopposite effect for poly (I:C) against the

different pathogens was observed. This might be explained by poly (I:C) activating an immune response that is appropriate for killing certain microbes, but not others.

The effect against other protozoan infections of *in vivo* treatment with exogenous type I IFNs has been reported. In the case of *L. major,* it was found thattreatment of highly susceptible BALB/c mice with a low (but not high) dose of IFN-β was protective against infection (Mattner *et al.,* 2004). Also, treatment with IFN-α prevented death caused by cerebral *P. berghei* malaria in C57BL/6 mice and reduced the development of the blood stage of infections (Vigario *et al.,* 2007). However, although a protective role for type I IFNs in immunity to *C. parvum* has been demonstrated in the present study, treatment of SCID mice with exogenous IFN-α did not cause any inhibition of infection. This negative result may have been obtained because of the route of administration of the cytokine or the dose of IFN-α used. Further *in vivo* studies are needed to explain this result.

In summary, the results presented in this chapter are the first to demonstrate a protective role for type I IFNs against *Cryptosporidium* infection. IFN-α/β wereexpressed by infected enterocytes *in vitro* and the cytokines directly inhibited parasite development. This suggested the possibility of the rapid establishment of an autocrine protective innate immune response. It was also shown with *in vivo* infections in neonatal SCID mice that IFN-α/β had a protective role that was evident early during infection and appeared to be independent of IFN-γ. Type I IFNs may therefore play a major part in the protective innate immune response that is important for controlling the reproduction of *C. parvum.*

CHAPTER FOUR

THE ROLE OF NK CELLS IN IMMUNITY TO *CRYPTOSPORIDIUM PARVUM*

4.1 GENERAL INTRODUCTION

4.1.1 NK cells

Natural Killer (NK) cells are key cellular mediators of innate immunity against intracellular pathogens and tumours (Crewenka and Lanier, 2001). They are a major source of IFN-γ (Lanier, 2008), a cytokine that plays an important part in immunity against *C. parvum* in mice (McDonald, 2000). In addition, NK cells appear to play an important part in the establishment of a T_H1 response against numerous infections, in part by their ability to produce IFN-γ (Zucchini, 2008). Until now the role of NK cells in immunity to cryptosporidia has been unclear. It was, therefore, of great interest to investigate closely the function of these cells in development of host resistance to *C. parvum*.

4.1.2 Biology of NK cells

NK cells are lymphocytes classically defined by the absence of the CD3 T cell marker and the presence of CD56 in human cells. The expression of other surface molecules such as leukocyte marker 7 (Leu-7 or CD57), IL- 2 receptor β chain (IL-2Rβ or CD122), FcγRII (CD16) and killer cell lectin-like receptor B1 (KLRB1 or CD161) has also been used as markers (Zucchini, 2008). In mice, NK cells are commonly recognised by the expression of integrin subunit α2 (DX5 or CD49b), asialo-ganglioside M1 (ASGM1) and natural killer receptor P1C (NKR-P1C or NK 1.1) (Korbel *et al.,* 2004). Antibodies to these latter markers are commonly used for NK cell depletion in mice. Both in humans and mice, many of those molecules are however expressed on other cells (Korbel *et al.,* 2004). Recently, the NKp46 molecule has been identified as the most specific NK cell marker in mammals (Walzer *et al.,* 2007).

Human subsets of NK cells can be distinguished by the surface density expression of the CD56 antigen (i.e., in flow cytometry, $CD56^{bright}$ and $CD56^{dim}$) (Papamichail, *et al.*, 2004). Resting $CD56^{dim}$ cells (comprising about 90% of total NK cells) are the most cytotoxic subset (Robertson and Ritz, 1990). The $CD56^{bright}$ NK cell subset (representing about 10% of NK cells) is linked to the high level of production of IFN-γ, TNF-α, TNF-β, GM-CSF and IL-10 upon activation while the $CD56^{dim}$ cells produce only small amounts of these cytokines (Robertson and Ritz, 1990). In addition, $CD56^{bright}$ cells express high levels of the C-type lectin-like CD94/NKG2 family with only small levels of killer-cell immunoglobulin-like receptors (KIR). $CD56^{dim}$ NK cells express both KIR and C-type lectin receptors at high density (Farag *et al.,* 2002).

The natural killer T (NKT) cells represent a lymphocyte subpopulation that expresses both CD56 and CD3-T cell receptor (TCR) complex (Bendelac *et al.,* 1997). NKT cells are highly restricted in antigen recognition capacity through the TCR and lipids appear to be what is mainly recognised. They share functions and receptors with NK cells. NKT cells possess an activation or memory phenotype by the expression of CD44, CD69 and CD122. They are present in most tissues where T cells are found (Papanichail *et al.,* 2004). NKT cells are capable of producing large amounts of T_H1 or T_H2 cytokines (Zlotnik *et al.,* 1992) and can also mediate lysis of classical NK cell targets (Koyasu *et al.,* 1992).

4.1.3 Development of NK cells

In vivo, NK cells have a limited life span, and in homeostasis the population must be continually replenished to maintain biological significance (Yokoyama *et al.,* 2004). NK cell development primarily occurs within the bone marrow. In humans, $CD34^+$ hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs) including a

small percentage of NK cell precursors (pre-NK) are normally enriched within the bone marrow (Srour *et al.,* 1991). Many factors are involved in the differentiation of NK cells, including cytokines, membrane factors, transcription factors and the microenvironment of the bone marrow. Defining the stages of differentiation of NK cells depends on the surface markers of NK cells (Yoon *et al.*, 2007).

IL-15 bound to dendritic cells (DCs) via IL-15Rα is considered an essential factor during NK cell development (Ferluzzo and *et al.,* 2009). It stimulates NK cell proliferation (Budgaian *et al.,* 2006; Casron *et al.,* 1994) and survival (Huntington *et al.,* 2007) and primes NK cell responses (Lucas *et al.,* 2007). Despite this it was observed that IL-15 was not required for the transition from HSCs to precursor NK cells (Vosshenrich *et al.,* 2005). In humans, IL-2 promotes the development of certain receptors on developing NK cells (Ferlazzo *et al.* 2004). In mice, IL-2 may not be necessary for the development of NK cells as normal populations of NK cells are found in IL-2 deficient mice (Liu *et al.*, 2000).

4.1.4 NK-like cells

In addition to NK cells, further innate lymphocyte subsets have also been described recently. These cells are components of lymphoid tissue inducer (LTi) cells. During embryonic development, LTi cells are crucial for lymphorganogenesis (Sun *et al.*, 2000). Lymphocytes phenotypically resembling LTi cells can also be identified after birth but their role is not well defined. LTi-like cells within the intestinal lamina propria of adult mice serve as inducer cells of tertiary lymphoid organs such as cryptopatches and intestinal lymphoid follicles (Bouskra *et al.*, 2008).

Recent studies have highlighted the presence of a population of IL-22-producing lymphocytes in the gut. They are identified by their co-expression of the retinoic acid

receptor-related orphan receptor-γt (RORãt) and the activating natural killer receptors (NKRs) (i.e. NKp46^{+}RORãt^{+} cells).

Unlike conventional NK cells, which depend on IL-15 for their development, the differentiation of NKp46^{+}RORãt^{+} cells is dependent on RORγ (Sanos *et al.,* 2009; Luci *et al.,* 2009).

Although these cells share characteristics of both NK and LTi cells and express various stimulatory NK cell receptors such as NKp46 and NKG2D, they differ from conventional NK cells in that they have only intermediate expression of NK1.1 and they lack the cytotoxic and IFN-γ production functions that typical NK cells are characterized by (Ferlazzo and Munz, 2004; Cella *et al.,* 2009). Instead, the NKp46^{+}RORãt^{+} cells are recognized for their ability to selectively produce IL-22 and therefore these cells are sometimes referred to as NK-22. Stimulation with IL-23 further boosts the production of IL-22, (Cella *et al.,* 2009). Importantly, IL-22 in the gut and other mucosal surfaces protects epithelial barrier function and activates antimicrobial defence of epithelial cells against invading pathogens (Ouyang and Valder2008).

Another important innate lymphocyte subset in the gut is a population of RORãt^{-}NKR^{-} LTi cells which is the dominating subset of NKR-LTi cells in the colon. They express NKp46 but lack RORγt. Colonic RORãt^{-}NKR^{-}LTi cells resemble conventional NK cells but they differ in their cytokine profile. They retain IL-23 receptor expression and interestingly, these cells but not RORãt^{+} NKR^{-}LTi, LTi or conventional NK cells produce IFN-ã in response to IL-23 (Vonarbourg*et al.,* 2010).

4.1.5 Activating and inhibitory receptors of NK cells

NK cells express a range of activating and inhibitory receptors. These receptors provide signals the balance of which decides whether NK cells become activated or remain inactivated. This helps regulate NK cell functions, ensuring protection against infected or malignant cells, yet preventing NK cell-driven autoimmune reactions.

4.1.5.1 NK cell signalling pathways

NK cell receptors use opposing signalling pathways to stimulate or inhibit activation. The inhibitory receptors signal via the intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs), located in the cytoplasmic tail of these receptors. ITIM-mediated signals result in both dephosphorylation and phosphosphorylation of intracellular components. The inhibitory receptors commonly recruit tyrosine phosphotases (SHP-1 or SHP-2) that help to interfere with the adhesion of NK cells to their target cells and suppress NK cell responses by dephosphorylating the protein substrates of the tyrosine kinases linked to activating NK receptors (Ravetch and Lanier, 2000). The activating receptors signal either through the immuno-receptor tyrosine based activating motifs (ITAMs) or through alternative signalling mechanisms using adaptor molecules DAP-10 or DAP-12. The ITAMs are non-covalently associated molecules and not in the receptors' cytoplasmic tail. After phosphorylation of a tyrosine residue in the tail, kinases are recruited leading to degranulation and transcription of cytokine and chemokine genes (Tomasello *et al.,* 2000).

4.1.5.2 Ly49 family receptors

The C-type lectin-like Ly49 receptors represent a large family of receptors in mice. The majority are inhibitory receptors which signal through an ITIM, while activating Ly49

receptors use the DAP-12 molecule for signalling (Smith *et al.,* 1998). Ly49 receptors recognise mainly MHC class I molecules or related proteins.

4.1.5.3 KIR family receptors

In humans and primates, the killer immunoglobulin-like receptors (KIRs) replace the Ly49 family of mice. Members of this family can be inhibitory or activating and signal through the ITIM and DAP-12 ITAM pathways, respectively, and they both recognise MHC class I molecules (Pegram *et al.*, 2011). KIRs specifically bind HLA-A-B and –C molecules and recognise polymorphisms in these class I molecules. KIR receptors are also thought to play a role in the induction of NK cell tolerance for self tissue, although the mechanism involved is still unclear (Pegram *et al.*, 2011).

4.1.5.4 CD94b-NKG2 heterodimer receptors

The CD94-NKG2A/C/E receptors belonging to the C-type lectin family are present in both humans and mice. These receptors react with the non–classical MHC molecules expressed on the surface of target cells and are described as being important in the prevention of inappropriate NK cell activation (Borrego *et al.,* 1998).

4.1.5.5 NKG2D receptor

Almost all NK cells express the NKG2D receptor that recognises cell surface stress molecules (Jamieson *et al.,* 2002). In the mouse, NKG2D signals by recruiting DAP-10 or DAP-12 molecules (Wu *et al.,.* 1999), while in humans NKG2D is associated with the DAP-10 signal pathway cytotoxicity and cytokine responses (Wu *et al.,.* 1999; Billadeau *et al.,* 2003). Evidence indicates that NKG2D is important in the NK cell-mediated control of some cancers, as it plays a role in the induction of cytotoxic, T_H1 and T_H2 responses (Diefenbach *et al.,* 2001; Westwood *et al.,* 2004). The ligands of the NKG2D receptor include MHC class I-related proteins whose expression is regulated by both

DNA damage and heat shock response pathways, both of which are often activated in tumours (Jolly and Morimoto, 2000). In human NK cells ligands for NKG2D include the stress related proteins MIC-A and MIC-B and ULBP1, ULBP2, ULBP3 and ULBP4 (Pegram *et al.,* 2011). Expression of MIC-A and MIC-B has been shown to be induced upon malignant transformation as a result of DNA damage (Jinushi *et al.,* 2003).

Although NKG2D has an important role in the immune response to tumours, several tumours have developed strategies to avoid this effect. For example, tumours have been reported to secrete NKG2D ligands, such as MIC-A (Groh *et al.,* 2002). Another mechanism of evasion by tumour cells is the secretion of TGF-β1 that down-regulates NKG2D on NK cells (Castriconi *et al.,* 2003).

4.1.5.6 Natural cytotoxicity receptors

The natural cytotoxicity receptors (NCRs) are a group of activating receptors that belong to the Ig-superfamily (McQueen and Parham, 2002). Resting and activated human NK cells express NCRs NKp46, NKp80, NKp30, while NCR NKp44 is only up-regulated upon stimulation of NK cells with IL-2 (Fuchs *et al.,* 2005). The NCRs have been found to be one of the main mechanisms by which NK cells kill tumours since deletion of single NCRs reduces the ability of NK cells to lyse tumour cells *in vivo* (Sivori *et al.,* 1999; Halfteck *et al.,* 2009). NKp30 has also been shown to be involved in NK cell-DC interactions, producing NK cell-mediated apoptosis of DCs (Moretta *et al.,* 2002).

4.1.6 Functions of natural killer cells

NK cells represent a major innate cellular component in the defence against stressed cells, microbe-infected or malignant cells. Their direct effector functions can be

subdivided into cytotoxic and cytokine production, mainly IFN-γ and TNF-α, as well as the secretion of numerous chemokines. NK cells also exert immunoregulatory functions including the modulation of DCs numbers and T cell responses. More recently NK cells were shown to take a memory-like function.

4.1.6.1 Direct cytotoxic effect

NK cells play a major role in the control of the replication of a variety of microbial pathogens, particularly viruses. This antimicrobial effect can occur through cytotoxic activity leading to the death of target cells through apoptosis via signalling pathways which depend on the receptors that are recruited on NK cells (Smyth *et al.,* 2005). Different killing mechanisms are employed by NK cells, including perforin/granzyme granule-mediated exocytosis, (Loh *et al.,* 2005). Signalling through the TNF-related apoptosis-including ligand (TRAIL) was also demonstrated, as in the case of encephalomyocarditis virus (Sato *et al.,* 2001), and through the Fas-L/Fas interaction as proposed for red blood cells infected by *Plasmodium falciparum* (Mavoungou *et al.,* 2003). NK cells are capable of inducing Fas expression on tumour cells *in vivo* via IFN-γ secretion then killing them in a Fas-L dependent manner (Bradley *et al.,* 1998; Screpanti *et al.,* 2001).

4.1.6.2 Cytokine secretion

NK cells can also make cytokines to help them achieve their immune effector functions including antimicrobial, anti-tumour and immunoregulatory functions. The main cytokine produced by NK cells is IFN-γ (Biron *et al.,* 1999). The first model system that described this protective activity in detail was demonstrated in studies with SCID mice infected with *Listeria monocytogenes* (Bancroft *et al.,* 1989). NK cell-derived IFN-γ helps enforce antimicrobial defences of cells in infected tissues and takes on increased

importance in the absence of cytotoxicity function (Presti *et al.,* 2001; Willberg *et al.,* 2007). This IFN-γ also contributes to the NK cell mediated anti-tumour activity such as by restricting tumour angiogenesis and stimulating further adaptive responses (Smyth *et al.,* 2005). TNF-α can also be released by NK cells, however other cell types can readily make this factor early during infections (Bancroft et al., 1989). Recently, results from both *in vitro* and *in vivo* studies demonstrated that NK cells are capable of the production of immunoregulatory cytokines including IL-10 (Bodas *et al.,* 2006; Grant *et al.,* 2008 Maroof *et al.,* 2008).

4.1.6.3 Chemokines production by NK cells

NK cells are also capable of expression of chemokines that have pro-inflammatory functions. *In vitro* studies showed that human peripheral NK cells cultured in the absence of stimuli can produce CCL4, CCL5 and CCL22 (Robertson, 2002). NK cells expressed CCL3 mRNA (Oliva *et al.,* 1998), but produced only small a amount of CCL3 protein (Fehniger *et al.,* 1998). Saito *et al.* also observed that $CD56^{bright}CD16^{-}$ NK cells which were isolated from human uterine deciduae expressed CXCL8 mRNA and secreted CXCL8 protein (Saito *et al.,* 1994). Greater amounts of these chemokines were detected after *in vitro* activation of NK cells. CXCL1, CCL1 and CCL3 were also produced (Robertson, 2002). Supernatants from activated NK cells stimulated the *in vitro* migration of $CD4^{+}$ T cells, $CD8^{+}$ T cells and neutrophils due to the production of chemokines (Somersalo *et al.,* 1994). IL-2 stimulated NK cells induced the chemotaxis of other NK cells (Nieto *et al.,* 1998).

4.1.6.4 Immunoregulatory functions

NK cells exert a variety of immunoregulatory effects that can contribute to promoting health over disease. Thus, activated NK cells can induce DC maturation (Gerosa *et al.,*

2002) and promote priming and expansion of $CD8^+$ T cells (Adam *et al.,* 2005). In addition, *in vitro* studiesindicate NK cells can also kill autologous immature myeloid DCs (myeloid DCs) via NKp30, NKp46 and DNAM-1 mediated recognition (Spaggiari *et al.* 2001) NK cell-mediated cell lysis was avoided by the up-regulation of MHC class I molecules, mainly HLA-E, ahead of DC maturation. Hence, NK cell' expression of an inhibitory receptor for HLA-E recognition is particularly important in modulating immature DC numbers (Della Chiesa *et al.,* 2003). In addition to myeloid DCs, activated macrophages were susceptible to NK cell-dependent cytotoxicity via NKG2D (Nedvetzki *et al.,* 2007). This cytotoxic activity reduces the population of antigen presenting cells for T cell activation and so limits the immune responses (Lunemann *et al.,* 2009).

NK cells are also important for promoting T_H1 polarization of $CD4^+$ T cells through the production of IFN-γ that initiates and implements T_H1 differentiation. This was detected in allogeneic immune responses during which NK cells produced high levels of IFN-γ that were adequate to mediate T cell polarization either by acting directly on naïve T cells or by enhancing DC maturation (Morandi *et al.,* 2006). Through the expression of OX40 ligand and CD86 upon ligation of the activating FcRIII (CD16), NK cells can induce IFN-γ production and proliferation of autologous T cells (Zingoni *et al.,* 2004). Other studies have demonstrated that NK cells are also capable of inhibiting T cell responses. *In vitro* studies showed that following T cell activation, T cells up-regulate NKG2D ligands and therefore became a target for NK cell-mediated cell lysis (Cerboni *et al.,* 2007; Roy *et al.,* 2008). Thus NK cells participate in a range of immunoregulatory functions through their effects on dendritic and T cells.

4.1.6.5 Memory-like function of NK cells

Immunological memory has two primary features: 1) antigen specificity and 2) expanded response following subsequent antigen exposure (Cooper *et al.,* 2009). Both T and B cells are known to express clonally unlimited numbers of antigen receptors that can recognize foreign antigens. After antigen stimulation and clonal expansion of T and B cells, a specific population of memory lymphocytes helps to protect the host from subsequent encounters with the same antigen (Murphy *et al.,* 2007). It was previously believed that innate immune cells lacked a memory component that was reactivated after repeated antigenic stimulation. However, recent studies have suggested that NK cells have memory-like properties (O'Leary *et al.,* 2006; Sun *et al.,* 2009). Different groups described, using murine models of contact hypersensitivity in response to a hapten, NK cell participation in a memory function previously attributed only to T and B cells. Contact hypersensitivity responses were observed in SCID and Rag-2-deficient mice which lack both T and B cells. However, in mice that lacked NK cells as well as T and B cells, contact hypersensitivity responses were absent. Furthermore, the adoptive transfer of NK cells from hapten-sensitized mice into naïve mice resulted in a delayed-type hypersensitivity reaction when recipients were challenged with the original hapten, but not when a different hapten was applied (O'Leary *et al.,* 2006). Hapten-experienced NK cells persisted for more than a month which is relatively longer than the normal turnover of NK cells which is 7-17 days (Koka *et al.,* 2003). The receptors responsible for hapten recognition by NK cells are not known (Held *et al.,* 2011). In murine cytomegalovirus (MCMV) infection, $Ly49^+$ NK cells showed changes compatible with memory, including longevity, improved cytolytic and cytokine production function as well as strong recall expansion (Sun *et al.,* 2009). In addition to antigen-driven memory, *in vitro* stimulation of NK cells with IL-12 and IL-18 followed by adoptive transfer into

naïve mice increased the NK cell lifespan and the ability to produce cytokines. It was also shown that these NK cells proliferated *in vivo* and their daughter cells also had a similar memory-like phenotype despite never having been activated (Cooper *et al.,* 2009). Collectively, these studies provide evidence that NK cells can have characteristics of memory, although underlying mechanisms are elusive.

4.1.7 Conditions of NK cell activation

NK cell activation in response to various infections (viral, bacterial, protozoal), seems to be under the control of different chemokines and cytokines secreted by macrophages and DCs as well as the direct interaction between these cells and NK cells (Zucchini *et al.,* 2008). These pro-inflammatory molecules differentially promote the expression of activation markers on NK cells that contribute to IFN-γ production and/or trigger NK cell-mediated cytotoxicity (Chiesa *et al.,* 2006; Dorner *et al.,* 2004).

4.1.7.1 NK cell activation by DCs

There has been a wealth of studies that have looked at NK cell-DC interaction resulting in cellular activation, maturation and even death (Cooper *et al.,* 2004) and in order for NK cell-DC interaction to occur *in vivo*, and to achieve optimal co-stimulatory effect, these cells must be recruited in close contact (Piccioli *et al.,* 2002). Sites of inflammation and lymph nodes were described as the main locations for such cross-talk (Cooper *et al.,* 2004). NK cells can be efficiently activated by DCs to obtain antitumor responses in mice (Fernandez *et al.,* 1999), to stimulate IFN-γ production, proliferation and cytotoxicty (Moretta, 2002; Ferlazzo and Mùnz, 2009).

DCs can directly activate NK cells by the secretion of different cytokines such as IL-12, IL-1, IL-18, IL-15 and type I IFNs (Ferlazzo and Mùnz, 2009). In the lymph nodes, DCs can also indirectly activate NK cells by enhancing the expansion of T cells which

secrete IL-2, that in turn stimulate NK cells (Fehniger *et al.,* 2003). Both DCs and T cells can activate NK cells by the up-regulation of ligands for stimulatory and co-stimulatory molecules expressed on NK cells (Cooper *et al.,* 2004).

4.1.7.2 The effect of cytokines

Different cytokines affect the expansion and modulate the effector functions of mature NK cells. IL-12 has been shown to play a central role in NK cell stimulation and the production of IFN-γ which in turn activates antimicrobial responses against *Listeria monocytogenes* (Tripp *et al.,* 1993), *Toxoplasma gondii*, *Trypanosoma cruzi* (Gazzinelli *et al.,* 1993; Cardillo *et al.,* 1996) and *Entamoeba histolytica* (Seydel *et al.,* 2000). Lieberman and Hunter demonstrated that IL-12 signalling pathways stimulate NK cells primarily through activation of the STAT4 pathway (Liberman and Hunter, 2002). Although IL-12 alone can stimulate IFN-γ production by NK cells, the related pro-inflammatory cytokines, IL-1 and IL-18, can both enhance this action *in vitro* (Hunter *et al.,* 1995; Micallef *et al.,* 1996) and in infection with *Cryptococcus neoformans* IL-18 stimulated NK cells to produce IFN-γ independently of IL-12 (Kawakami *et al.,* 2000).

In addition to the IL-12/STAT4 pathway for activation of NK cell responses, other cytokines and pathways have been shown to act as potent inducers of NK cell activation and IFN-γ secretion. Both IL-2 and IL-15 that share two of three receptor chains, are important for NK cell development and signalling through STAT3/STAT5 pathways they stimulate production of high levels of IFN-γ independently of IL-12, and can act synergistically with IL-12, IL-1, IL-18 or TNF-α (Lieberman and Hunter, 2002). Although it is well known that type I IFNs primarily signal via a JAK/STAT pathway activating STAT1 and STAT2, evidence indicates that those cytokines can also enhance NK cell production of IFN-γ via the phosphorylation of the STAT4 pathway (Hunter *et al.,* 1997; Lieberman and Hunter, 2002).

Type I IFNs have also been shown to enhance NK cell cytotoxicty as seen in infection with MCMV (Nguyen *et al.* 2002). NK cell activation by type I IFNs could either be via the direct effect of the cytokine on NK cells as in the case of vaccinia infection (Martinez *et al.,* 2008) or it can be due to the activation of DCs and/or macrophages for IL-15 production that promotes NK cell proliferation (Lucas *et al.,* 2007). A recent study has shown that supernatants of *Salmonella*-infected macrophages induce IFN-γ production in human $CD56^{+}$ NK cells and this induction of IFN-γ was critically dependent on IL-23 and IL-1β (van de Wetering *et al.,* 2009), indicating that IL-23 is another cytokine that stimulates NK cell activity.

IL-21 which is closely related to IL-2 and IL-15 can enhance NK cell cytotoxic activity and IFN-γ production (Parrish-Novak *et al.,* 2000). However, IL-21 does not stimulate NK cell proliferation and can act as an antagonist of IL-15-induced proliferation of those cells, thus limiting their duration of survival (Kasaian *et al.,* 2002). This could explain the rapid fall in NK cell activity following their initial peak of activity noticed in the course of many parasitic infections (Lieberman and Hunter, 2002). Other cytokines, but mainly TGF-β, contribute to the inhibition of NK cell responses (Bellone *et al.,* 1995). IL-10 has also been shown to have an inhibitory action on NK cell function (Gazzinelli *et al.,* 1992). This however is rather more complex as stimulatory effects have also been recognised with this cytokine Thus, IL-10 can inhibit the antimicrobial effects of IFN-γ (Gazzinelli *et al.,* 1992) as well as the production of other cytokines associated with NK cell development and IFN-γ release (Moore *et al.,* 2001). On the other hand, other studies indicate that IL-10 has stimulatory effects on NK cell proliferation and cytotoxicty when acting alone (Schwarz *et al.,* 1994) or in combination with other cytokines such as IL-18 (Cai *et al.,* 1999).

4.1.7.3 The effect of chemokines

Different chemokines have been shown to stimulate the migration of NK cells as shown by *in vitro* chemotaxis assays (Robertson *et al.,* 2002). Studies indicated that resting human NK cells migrate in response to known ligands for CXCR3 (CXCL9, CXCL10 and CXCL11) and CXCR4 (CXCL12) (Campbell *et al.,* 2001; Campbell *et al.,* 1998; Kim *et al.,* 1999; Romagnani *et al.,* 1996). Furthermore, it was also demonstrated that resting NK cells also migrate in response to CC chemokines, including CCL2, CCL3, CCL4, CCL5, CCL7 and CCL8 (Inngjerdingen *et al.,* 2001; Taub *et al.,* 1995; Allavena *et al.,* 1994; Drake *et al.,* 2001). Studies have also shown that chemokines may increase NK cell lysis of target cells by promoting the cytotoxic granule release by NK cells (Taub *et al.,* 1995; Yoeneda *et al.,* 2000). Soluble and membrane-bound CX3CL1 was also found to induce IFN-γ production by NK cells and affect NK cell ability to kill tumor cells both in vitro and in vivo (Guo *et al.,* 2003; Yoneda *et al.,* 2003).

4.1.8 Location and organ-specific features of NK cells

The distribution of NK cells from the bone marrow through the blood to the target organs is not static because these cells can re-circulate between different organs (Hoglund and Brodin, 2010). NK cells can react to a wide range of chemokines produced by cells that are specific to certain organs and thus recruit to different sites of inflammation (Gregoire *et al.,* 2007). The influence of organ-specific chemokines on NK cell trafficking suggests that organ-intrinsic elements may be required for NK cell homing during physiological and pathological conditions. Those elements may include the unique cellular components of each organ, the soluble components and the anatomical constituents (Shi *et al.,* 2011).

4.1.8.1 Gut NK cells

Many studies that investigated NK cells in the mucosal tissues were difficult to analyse due to the fact that it was difficult to distinguish bona fide NK cells from NKT cells and other populations of innate lymphoid cells that are common in the gut lymphoid tissue (Shi *et al.,* 2011). In the intestine, NK cells are found predominantly within the lamina propria and are rarely in lymphoid aggregates, although they can be found in parafollicular regions such as in caecal lymphoid patches, Peyer's patches and mesenteric lymph nodes (Reynders *et al.,* 2011).

4.1.9 NK cells in antiviral defence

Although it is now accepted that NK cells may provide a first line defense against different microbial pathogens, their close involvement in immunity against infection was first clearly established for many viral infections. In viral infections NK cells protective activity can be by either cell–mediated cytotoxicity or by IFN-γ production or both. In particular, the role of NK cell in immunity to herpes virus in both humans and mice is well understood.

4.1.9.1 NK cell in immunity to cytomegaloviruses

In MCMV infection NK cell based immunity involves both IFN-γ and direct lysis of infected cells (Lodoen and Lanier, 2006). NK cell mechanisms for the control of MCMV infection are variable in different mice strains, however (Vivier *et al.,* 2008). In C57/BL6 mice, NK cells can recognise MCMV virus through the interaction between a virus-encoded cell surface molecule, m157, and the NK cell receptor Ly49H (Smith *et al.,* 2002). Ligation of Ly49H by m157 during MCMV infection leads to the production of different cytokines and chemokines including IFN-γ, MIP-1α, MIP-β, RANTES and ATAC (Dorner *et al.,* 2004). In MCMV-resistant Ly49H$^-$ mouse strains, other NK cell

receptor-ligands participate in the recognition of infected cells by NK cells (Vivier *et al.,* 2008).

Studies also highlighted the interaction between NK cells and DCs (Andrews *et al.,* 2003). DCs express Toll-like receptors and secrete cytokines in response to microbes. In MCMV-infected C57BL/6 mice, the recruitment of NK cells to the sites of infection requires the production by local cells of IFN-α which then induces macrophages to express different chemokines that attract NK cells (Hokness *et al.,* 2005). Type I IFNs also induce DCs to produce IL-15 which is an important factor for NK cell-mediated control of MCMV. Studies with knockout mice that lack TLR9 and MyD88 pathway have shown that these mice produced reduced amounts of type I IFNs, IL-12 and IFN-γ in their sera in response to MCMV infection (Krug *et al.,* 2005). NK cells from these mice produced less IFN-γ, had impaired proliferation and exhibited reduced cytotoxic activity in comparison to NK cells from infected wild type mice (Krug *et al.,* 2005). Infection of DCs with MCMV also induced the DCs to secrete IL-12 and IL-18 that stimulated IFN-γ production by NK cells (Andrews *et al.,* 2003). Figure 30 summarises the interaction between DCs and NK cells in response to MCMV virus.

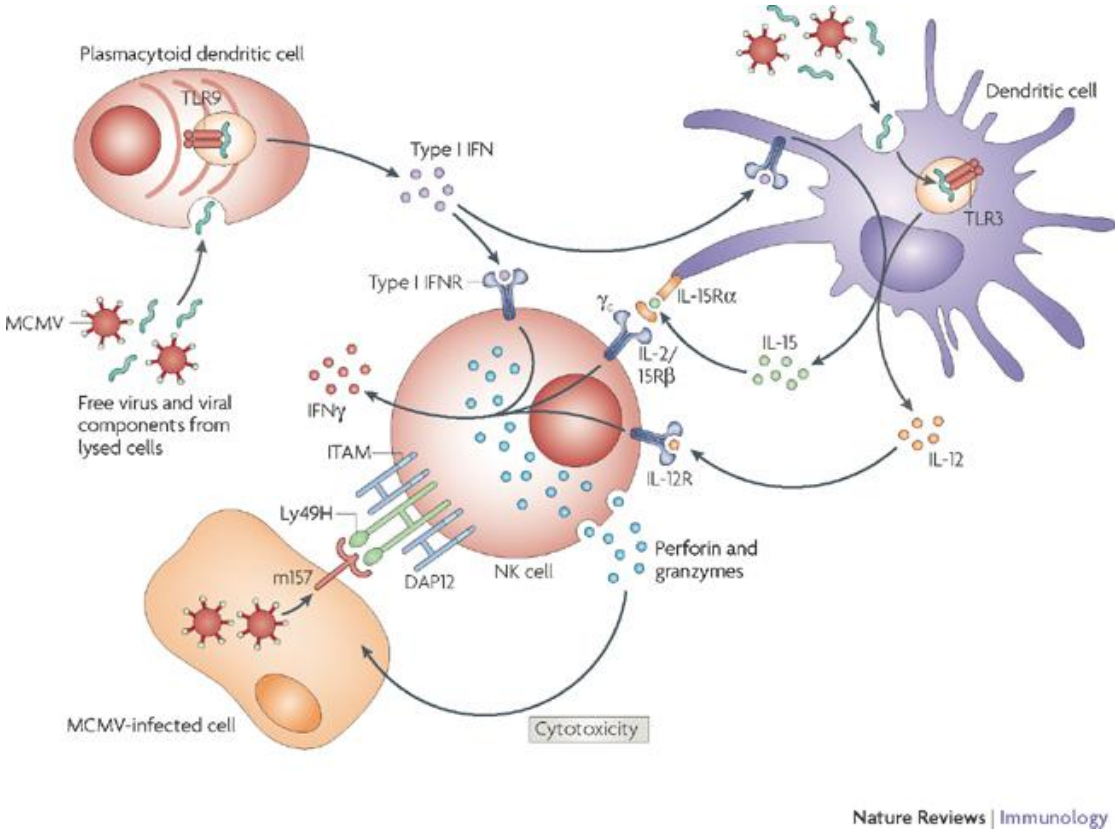

Figure 30. Interaction between DCs and NK cells during MCMV infection

Lanier LL, Nature Reviews (2008).

Infection with MCMV triggers the production of type I IFNs by pDC through TLR9 and the production of cytokines such as IL-12 by DCs via TLR3, as well as type IFN-induced production of IL-15 by DCs. IL-15 is presented to NK cells by the IL-15 receptor (IL-15Rα) on the surface of DCs, leading to NK cell activation. IL-12 together with IL-15 and potentially other pro-inflammatory cytokines, induces the secretion of IFN-γ by NK cells.

4.1.9.2 NK cells in HIV infection

The pathogenesis of human immunodeficiency virus infection (HIV) and acquired immunodeficiency syndrome (AIDS) is considered multifactorial in that no specific immune alteration has been identified that can fully explain the excess of dysregulations described so far (Boasso *et al.* 2008). Both the innate and acquired arms of immune responses are involved in the control of the infection. The role of NK cells in innate immunity has been characterised and was extensively reviewed by Alter and Altfeld, 2009. HIV infection is associated with significant changes in NK cell subset distribution in the peripheral circulation (Alter *et al.,* 2005). A dramatic reduction of $CD3^-$ $CD56^+$ cells proportion was reported in several studies (Lucia *et al.,* 1997), this is partially related to the emergence of the $CD3^-CD56^-CD16^+$ population, a subset of NK cells which is rare in healthy individuals (Alter *et al.,* 2005) and lack NK cell effector function (Mavilio *et al.*, 2006) This may explain the loss of NK cell function observed over the course of HIV-1 infection (Alter and Altfed, 2008).

Several studies have described the impact of specific HLA class I alleles on HIV-1 disease progression (Carrington and O'Brien,2003). The collected data indicates that HLA-B alleles in conjunction with KIR3DL1 are responsible for the greatest level of control over HIV-1 viral replication (Kiepiela *et al.,* 2004; Martin *et al.*, 2002; Martin *et al.,* 2007).

HIV-1 virus has developed an elegant way to evade NK cell antiviral mechanisms. This is achieved by the expression of HIV-1 Nef protein which strongly downregulates HLA-A, partially downregulates HLA-B and spares HLA-C. HLA-B and HLA-C serve as the primary ligands for inhibitory NK cells receptors (Le Gall *et al.,* 1998; Cohen *et al.,* 1999). Nef proteins also decrease the expression of the ligands for NKG2D receptor (Cerboni *et al.,* 2007).

4.1.10 NK cells in immunity to bacterial pathogens

4.1.10.1 The role of NK cells in infection with *Listeria*

Many studies indicate that NK cells play a protective role during the early phases of *L. monocytogenes* infection by producing IFN-γ for the activation of macrophage effector functions. Initial observations came from studies with SCID mice. For a few days SCID mice controlled the *Listeria* infection as efficiently as normal animals but later failed to obtain sterile immunity. Neutralization of IFN-γ with specific antibodies in SCID mice led to increased bacterial multiplication and it was suggested that NK cell production of IFN-γ is a major protective factor in the early phase of *Listeria* infection (Bancroft *et al.,* 1987; Bancroft *et al.,* 1991). Another study using Rag $2^{-/-}\gamma c^{-/-}$ confirmed the importance of NK cells in the resistance to listeriosis as these mice succumbed to *Listeria* infection with the same pathophysiology as IFN-γR-deficient mice unlike Rag $2^{-/-}$ mice that survived (Andersson *et al.,* 1998). Others showed that IFN-γ production 24h postinfection in C57Bl/6 mice was abrogated *in vitro* and *in vivo* by depletion of $NK1.1^{+}$cells with anti-NK1.1 mAbs, indicating that $NK1.1^{+}$cells are major IFN-γ producers at day 1 (Teixeira and Kaufmann,1994). The *in vitro* production of IFN-γ by SCID splenocytes was shown to be IL-12 dependant and TNF-α was required as aco-stimulating factor (Tripp *et al.,* 1993).

4.1.10.2 The role of NK cells in infection with *Mycobacterium tuberculosis* and *M. avium*

NK cells purified from mouse spleen cells were shown to lyse *M. avium*-infected monocytes, but not uninfected monocytes (Katz *et al.,* 1990). IL-2-activated mouse NK cells were found highly bactericidal against intracellular *M. avium* (Bermudez et al., 1991). Human NK cells have been shown to produce IFN-γ in cultures of mycobacterial

stimulated peripheral blood mononuclear cells (Gerosa *et al.,* 2002) and to mediate killing of *M. tuberculosis*-infected monocytes (Vankayalapati *et al.,* 2002).

Depletion of NK cells in wild type mice using anti-asialo GM1 antibody or anti-NK1.1 antibody in wild-type mice resulted in a significant increase in the growth of *M. avium* colonies in the spleen, suggesting a protective role for NK cells (Harshan and Gangadharam, 1991). However, other similar *in vivo* studies failed to show increased susceptibility to *M. avium* infection in immuncompetent mice (Sanders *et al.,* 1996; Florido *et al.,* 2003). Similarly, NK cell depletion in wild type mice had no effect on the *M. tuberculosis* bacterial load or on pathology (Junqueira-Kipnis *et al.,* 2003). In contrast to these studies, investigations with immunocompromised mice indicated strongly an important protective role for NK cells in the host response to mycobacteria. Thus, in *M. avium* infection, SCID mice formed protective hepatic granulomas and this response was dependent on IFN-γ and TNF-α expression by NK cells (Smith *et al.,* 1997). Moreover, TCRαβ$^{-/-}$ mice were found to survive longer than IFN-γ$^{-/-}$ mice after infection with *M. tuberculosis* (Mogues *et al.,* 2001).These findings suggested a T cell-independent source of IFN-γ and highlighted the involvement of NK cells in innate immunity to mycobacteria*.* In an extensive investigation of the role of NK cells in immunity to *M. tuberculosis* in Rag2$^{-/-}$ mice (Feng *et al.*, 2006) the main findings of this study were: 1) NK cells were the main source of IFN-γ and these mice were more resistant to infection than IFN-γ$^{-/-}$ mice or anti IFN-γ-treated Rag2$^{-/-}$ mice. 2) NK cell-deficient Rag2$^{-/-}$γc$^{-/-}$ mice were more susceptible to infection than Rag 2 $^{-/-}$ mice with the time to death in the former group being similar to that for IFN-γ$^{-/-}$ mice. 3) *In vitro* stimulation of Rag2$^{-/-}$γc$^{-/-}$ splenocytes with *M. tuberculosis* failed to induce the production of IFN-γ. 4) NK cell-mediated innate resistance depended on IL-12 and not IL-23. Furthermore, IFN-γ neutralization in Rag 2 $^{-/-}$ mice resulted in a loss of pulmonary expression of NOS2, a known critical mediator during macrophage activation. IFN-γ

production also regulated pulmonary inflammation during early infection. These findings indicate that NK cells are important in innate immunity to *Mycrobacterium* infection, particularly in immunocompromised hosts.

4.1.10.3. The role of NK cells in infection with invasive enteric bacteria

Shigellosis is an invasive disease of the human intestinal tract that represents a major cause of bacillary dysentery worldwide and of several species of *Shigella,*S. *flexneri* is one of the most important. Unfortunately adult mice are resistant to infection with *S. flexneri* by the gastric route, but an *in vivo* model of intranasal infection of mice with *S. flexneri* resulted in an inflammatory response similar to that during human intestinal infection in humans (Voino-Yasenetsky, 1962). IFN-γ was shown to be important in the control of infection as a higher rate of infection was detected in IFN-$\gamma^{-/-}$ mice than in wild type mice (Way *et al.*, 1998). A role for NK cells in immunity was suggested by the findings that greater levels of infection were obtained in SCID*beige* mice (with defective NK cell cytotoxicity, but also reduced neutrophil exocytosis) than in SCID mice, and also in wild type mice depleted of NK cells using anti-asialo-GM1 compared with untreated controls (Way *et al.,* 1998). Le-Barillec *et al.* demonstrated that both wild type and Rag2$^{-/-}$ mice controlled and survived the infection while NK cell deficient Rag2$^{-/-}\gamma c^{-/-}$ all died by 9 days postinfection and this was associated with relatively low expression of proinflammatory cytokines. However, when Rag2$^{-/-}\gamma c^{-/-}$ mice were reconstituted with TCR$\alpha\beta^{+}$ T cells from wild type mice they were as resistant to infection as wild type mice, but the same cells from IFN-$\gamma^{-/-}$ mice failed to enhance protection against *S. flexneri* (Le-Barillec *et al.,* 2005). These results demonstrate an important role for NK cells in innate immunity to *S. flexneri* by acting as a key source of IFN-γ in the early stage of infection.

Studies with *Salmonella* infection have also supported a beneficial role for NK cells in the innate immunity to this enteric pathogen. Strong resistance of hypersusceptible C3H/HeJ miceagainst virulent challenge with *Salmonella typhimurium* following immunisation with the avirulent SL3235 strain (Killar and Eisenstein, 1985) was associated with elevated numbers of NK cells in the spleens and peritoneal cavities of immunised mice (Schafer and Eisenstein, 1992). Furthermore, depletion of NK cells with anti-asialo GM-1 antibody increased mortality in mice challenged with the virulent strain (Schafer and Eisenstein, 1992). An important protective role for IL-15 against infection with avirulent *Salmonella choleraesuis* in wild-type mice was indicated by the high level of IL-15 produced during infection coincident with elevated number of NK cells. Also, treatment of mice with anti-IL-15 neutralising antibody prevented the increase in NK cell numbers and also reduced the level of IFN-ã in serum and led to a failure to clear bacteria (Hirose *et al.,* 1999). Early peroral infection with *Salmonella enterica serovar typhimurium*, resulted in significant up-regulation of IFN-ã and IFN-ã-associated chemokines (CCL10, CCL5, CXCL-9 and CXCL-11) as well a significant increase in NK cells in the gut. Furthermore, antibody-mediated depletion of NK cells in wild type mice or using T, B and NK cell-deficient mouse starin, resulted in significant reduction of IFN-ã expression in the intestine. In addition, *in vitro* stimulation of splenocytes with *Salmonella* resulted in significant IFN-ã production by NK cells. These observations support NK cells as a major cellular source of IFN-ã early during (Harrington *et al.,* 2007). Finally, supernatants of *Salmonella* infected human macrophages induced IFN-γ production in $CD56^{+}$ cells that was critically dependent on IL-23 and IL-1β (van de Wetering *et al.,* 2009).

4.1.11 NK cells in immunity to protozoan pathogens

Besides their major role in immunity to viral and bacterial infections, there is evidence that NK cells represent a major element in the innate immune response to a variety of protozoal infections.

4.1.11.1 The role of NK cells in infection with *Leishmania*

There is evidence for a protective role for NK cells in early resistance to *Leishmania major* infection since a higher number of parasites at the lesion site was found in NK cell-depleted mice (Laskay *et al.,* 1993). Also, NK cells were identified as the main source of IFN-γ required for $CD4^+$ T cell differentiation and in controlling early resistance to *L.major* (Scharton and Scott, 1993). *L. amazonensis* infection could not be completely controlled in the absence of NK cells (Laurenti *et al.,* 1999). Depletion of NK cells in SCID mice abolished their ability to control infection, suggesting a T cell-independent mechanism for the control of the infection (Laskay *et al.,* 1995). In a study of infection with *L. tropica* of *beige* mice with lymphocyte (including NK cell) cytotoxicity defect, the ability to control the infection was only slightly compromised suggesting that cytotoxicity was not an essential mechanism for resistance (Kirkpatrick and Farrell, 1983). *In vitro* studies, however, indicated that NK cells exhibited cytotoxicity activity in response to mucosal leishmaniasis, but that this activity was involved in tissue pathology rather than in protection (Brodskyn *et al.,* 1997). IL-2-activated NK cells (A-NK) display high cytotoxicity *in vitro* and can kill both NK cell-sensitive and -resistant targets (Trinchieri *et al.*, 1984). A study by Aranha *et al.* demonstrated that A-NK cells play a major role in the control of infection of macrophages via direct lysis of the host cell and/or parasites. This was supported by the reduction in the number of viable

parasites, increased parasite degeneration and cellular lysis occurring in the cultures of infected macrophages exposed to different numbers of A-NK cells (Aranha *et al.* 2005).

In humans, spontaneous healing from leishmaniasis due to *L. aethiopica* appeared to be related to the ability of NK cells to proliferate and produce cytokines (Massho *et al.,* 1998). Studies indicated that the activation of NK cells in leishmaniasis is a cytokine/chemokine-mediated rather than NK receptor-mediated. Both IL-12 and IL-18 were described to be important in NK cell activation in response to *L. major* infection (Scharton-Kersten *et al.,* 1995; Wei *et al.,* 1999). In visceral leishmaniasis, a 7-day treatment of BALB/c mice with IL-12 led to a 70% reduction of the liver parasite load compared with untreated control mice, whereas in IL-12–treated but NK cell–depleted mice the decrease of the parasite numbers was only 30% (Murray and Hariprashad, 1995). A deficiency in NK cell-activating chemokines resulted in suboptimal NK cell activity in response to *L. major* and the local treatment of BALB/c mice with recombinant chemokines shortly after infection resulted in an enhanced NK cell activity in the draining lymph node (Vester *et al.,* 1999). Recently, Haeberlein *et al.*demonstrated that IL-15 activity during *L. infantum* infection of mice was not required for IL-12-dependent stimulation of NK cell IFN-γ production and cytotoxicity. IL-18 also helped to trigger NK cell effector functions, but comparing the NK cell response in WT, IL-$12^{-/-}$ and IL-$18^{-/-}$ mice it was apparent that IL-18 was not essential for immunity. IL-18 was shown, however, to enhance the response of NK cells to IL-12 *in vivo* and *ex vivo*. Finally, in the absence of IL-18, IL-12 was capable of stimulating NK cell activity against *Leishmania* (Haeberlein *et al.*, 2010).

NK cell–DC interaction in response to *Leishmania* has also been described. IL-12 production by DCs in the first 24h of infection was required to trigger NK cell activation (Berberich *et al.,* 2003; Sher *et al.,* 2003). In an *in vivo* study, myeloid DCs were found

essential for obtaining NK cell cytotoxicity and IFN-γ release in vivo through the production of IL-12 and this was dependent on the upregulation of TLR9 on DCs (Schleicher *et al.,* 2007).Furthermore, Sanabria *et al.*found that the addition of resting NK cells significantly enhanced the activation of DCs preinfected with *L. amazonensis* promastigotes and that these activated DCs, in turn, stimulated NK cell activation mostly via cell contact-dependent mechanisms (Sanabria *et al.*, 2008).

Direct stimulation of NK cells in leishmaniasis has also been described. Live promastigotes of *L. donovani* and *L. aethiopica* activated purified NK cells to secrete IFN-γ in the absence of antigen presenting cells (Nylen *et al.*, 2003) and direct activation of TLR-2 on NK cells by *L. major* lipophosphoglycan (LPG) up-regulated TLR2 and increased expression of IFN-γ and TNF-α (Becker *et al.*, 2003).

4.1.11.2 The role of NK cells in infection with *Trypanosoma cruzi*

A number of studies have described a protective role for NKcells in the innate immunity against *Trypanosoma cruzi*, the agent of Chagas disease. Depletion of NK cells in infected BALB/c and C57BL/6 mice led to higher level of parasites, increased mortality and a delay of IFN-γ production by T cells (Rottenberg *et al.,* 1988; Une *et al.,* 2000). The control of parasitaemia in early infection was associated with the production of IFN-γ by IL-12 stimulated NK cells (Rottenberg *et al.,* 1988; Cardillo *et al.,* 1996, Gazzinelli *et al.,* 1993). Cytotoxic NK cells (or T cells) were found not to be essential for the control of *T. cruzi* in studies with mice having the beige mutation (Hatcher *et al.,* 1981). In agreement with this observation Une *et al.* demonstrated that in experiments with IFN-α/β-deficient mice that NK cell cytotoxicity was not crucial for the control of infection (Une *et al.*, 2003). In contrast, mice deficient in either perforin/granzyme or Fas/ FasL cytolytic pathways suffered from early death and these pathways were important for

parasite killing in tissues, implying a possible important role for cytotoxic NK cell in immunity (Muller *et al.,* 2003).

In humans, the importance of NK cells in resistance to Chagas disease has been illustrated in numerous studies and was recently reviewed by Sathler-Avelar *et al.,* 2009. Thus in the early acute human Chagas disease no changes in NK cells were observed (Sathler-Avelar *et al.,* 2003), but in the late acute phase of disease there was a selective increase in a specific lineage of NK cells ($CD16^{+}CD56^{-}$) (Sathler-Avelar *et al.,* 2009). Furthermore, it was demonstrated that the expansion of $CD16^{+}CD56^{-}$ pre-NK cells as well as a higher level of pro-inflammatory monocytes before the activation of T cell-mediated immunity is the main feature of the early indeterminate stage of Chagas disease. The expansion of pre-NK cells might suggest an important mechanism for macrophage activation via the production of IFN-γ (Vitelli-Avelar *et al.,* 2006). An *in vitro* cytokine analysis demonstrated that there was a shift in cytokine profile in NK cells upon stimulation with *T. cruzi* antigens leading to elevated levels of cells producing IFN-γ, TNF-α, but also IL-4. This may suggest that NK cells could provide protection against tissue damage caused by severe inflammation (Sathler-Avelar *et al.,* 2006). However the *ex vivo* cytokine profiles of circulating NK cells and monocytes during early indeterminate Chagas disease were similar to those observed in healthy uninfected children (Sathler-Avelar *et al.,* 2006). In the late chronic phase of the disease Vitelli-Avelar *et al.* observed that in all chronic Chagas disease patients there was an increased frequency of circulating NK cells and the percentage of $CD3^{-}CD16^{+}CD56^{+}$ and $CD3^{-}CD16^{+}CD56^{dim}$ NK cells was high in indeterminate patients. This suggested to the authors a possible protective role for these NK cells populations in controlling morbidity (Vitelli-Avelar *et al.,* 2005).

4.1.11.3 The role of NK cells in infection with *Toxoplasma gondii*

Early studies by Hauser *et al.* indicated that NK cells had enhanced cytotoxic activity against *T. gondii* in *in vitro* infected macrophages (Hauser *et al.,* 1982; Hauser and Tsai, 1986). Similar to the findings with *Leishmania,* NK cell cytotoxicity may have been of minor importance as no effect on survival was seen in studies with beige mice (Hughes *et al.,* 1988). The severe outcome of disease after NK cell depletion in *T. gondii*-infected mice suggested an important a protective function of NK cells other than cytotoxicity (Hunter *et al.,* 1995).

Both indirect and direct modes of activation of NK cells have been described. In an *in vitro* study NK cell activation was absolutely dependant on IL-12-producing macrophages (Gazzinelli *et al.,* 1993). Other reports also described a role for DCs in the recognition of *T. gondii* and the stimulation of NK cells (Sher *et al.,* 2003).

The effect of different cytokines on NK cell activity against *T. gondii* antigen *in vitro* has also been described. IL-12, IL-15 and TNF-α were involved in IFN-γ production by cultured splenocytes obtained from SCID mice, while IL-10 inhibited IFN-γ release (Hunter *et al.,* 1994). Further *in vivo* study revealed that intraperitoneal *T. gondii* infection of wild-type C57BL/6 mice and IL-$15^{-/-}$ mice produced similar serum levels of IFN-γ 7 days following infection and suggesting that IL-15 is not required for *in vivo* activation and expansion of NK cells (Lieberman *et al.*, 2004).

A study by Goldszmid *et al.* confirmed the importance of NK cells in immunity to *T. gondii* and showed that NK cell activity could promote the induction of an early protective $CD8^+$ T cell response in an IFN-γ-dependent matter (Goldszmid *et al.*, 2007).

Although NK cells are known for the production of proinflammatory cytokines, mainly IFN-γ, they are also capable of releasing IL-10 (Deniz *et al.,* 2008). In the case of

systemic toxoplasmosis, NK cells were the first and the most frequent IL-10-expressing cell population induced in non-lymphoid tissues. NK cell release of IL-10 acts to inhibit the pathogen-stimulated production of IL-12 by DCs and therefore appeared to participate in the immunosuppressive mechanisms of the infection (Perona Wright *et al.,* 2009)

4.1.11.4 The role of NK cells in infection with *Neospora caninum*

Neospora caninum is a relatively recently described intracellular apicomplexan parasite that is closely related to *T. gondii* (Dubey *et al.,* 2002) andis a major cause of abortion and congenital infection in cattle worldwide (Dubey *et al.,* 1996). Whereas oocysts shed in cat faeces is a major source of infection for *T. gondii,* oocysts from dog faeces infect cattle. It has been claimed that nonhuman primates have been experimentally infected leading to a disease that had notable similarities to toxoplasmosis in humans (Barr *et al.,* 1994). Cell-mediated immunity and IFN-γ are major components of immunity against this pathogen (Innes *et al.,* 2002, Innes *et al.,* 1995, Baszler *et al.,* 1999). Boysen *et al.* demonstrated an important role for NK cells in the immunity against this parasite. *In vitro* stimulation of NK cells with tachyzoites directly triggered the production of IFN-γ independently of IL-12 but expression could be enhanced by the addition of the cytokine. A similar response was detected in cocultures of NK cells and *N. caninum* infected fibroblasts. It was also shown that in culture, NK cells had increased cytotoxicity towards infected fibroblasts in comparison to uninfected fibroblasts, mainly by a perforin-mediated mechanism. Furthermore, this was the first study to describe the infection of NK cells with this intracellular parasite (Boysen *et al.,* 2005).

4.1.11.5 The role of NK cells in infection with *Plasmodium*

Studies with experimental mouse models and with human *P. falciparum* indicate a significant role for NK cells in the early control of infection and in establishment of the adaptive immune response (Roetynck *et al.,* 2006).

In studies with non-lethal *P. yoelii* and *P. chabaudi*,it was demonstrated that infection with parasitised red blood cells (pRBC) was associated with an early burst of IFN-γ activity 24 h after challenge. This response appeared to be partially dependent on NK cells, since immunocompetent mice depleted of NK cells by anti-asialo GM-1 (ASGM-1) antibody showed a reduced IFN-γ response 24h postinfection and failed to control the infection (Mohan *et al.,* 1997, De Souza *et al.,* 1997). Similar results were obtained in NK cell-depleted SCID mice using ASGM-1 antibody (Choudhury *et al.,* 2000) and these mice died much earlier than the control group (Choudhury *et al.,* 2000).

Infection of $Rag2^{-/-}$ and $Rag2^{-/-}\gamma c^{-/-}$ mice initiated with *P. yoelii* sporozoites confirmed a significant role for NK cells in resistance against malaria as the $Rag2^{-/-}$mice were more resistant. The same study demonstrated that NK cells were cytotoxic to YAC-1 tumour cells and pre-erythrocytic infectedliver cells stages *in vitro* but not to the erythrocytic stages. These findings suggested a protective role for NK cells in the pre-blood stage of infection with *P. yoelii* (Roland *et al.,* 2006).

In contrast to the earlier studies, Couper *et al.* found no clear role for either T cells, NK cells or IFN-γ in controlling the early *P*. *yoelii* infection. However, depletion of monocytes/macrophages exacerbated parasite growth and anaemia during both lethal and nonlethal acute *P*. *yoelii* infections, indicating that there is an IFN-γ-, NK cell- and T cell-independent pathway for induction of effector macrophages during acute malaria infection (Couper *et al.*,2007).

Studies with *P. falciparum* have suggested a protective the role for NK cells in human malaria. NK cell from the blood of patients were shown to be responsible for an IFN-γ response *in vitro* (Artavains-Tsakonas and Riley, 2002). This activation was mainly dependant on IL-12 and to a lesser extent on IL-18 (Artavains-Tsakonas and Riley, 2002). A study by Orago and Facer also found that purified NK cells from healthy and *P. falciparum*-infected individuals directly lyse parasititised erythrocytes *in vitro* (Orago and Facer, 1991). In an experimental study with immunised individuals, elevated levels of IFN-γ and soluble granzyme A were found at the time of parasite release from the liver into the circulation, suggesting the possible role for inflammatory cytokines and cytotoxicity in the initial defence against blood stage of infection (Hermsen *et al.,* 2003). Based on these findings, Korbel *et al.,* have suggested that the activation of human NK cells by blood stages of infection depended on at least two signals: cytokine release by accessory cells such as macrophages and DCs and by direct recognition of infected cells by NK cell receptors (Korbel *et al.,* 2004).

Thus NK cells seem to play a significant role in the early innate immune responses against different specious of *Plasmodium* via the production of IFN-γ and via the direct killing of target cells, although contradictory observations have also been made.

4.1.12 Clinical applications of NK cells and their role in some human non-infectious diseases and pregnancy

4.1.12.1 NK cells in cancers

Different *in vitro* studies from human and other mammalian species, as well as *in vivo* studies in mice and rats have provided evidence for involvement of NK cells in immunity against tumours (Trinchieri *et al.,* 1989). *In vivo* murine studies using antibodies to deplete NK cells have supported an anti-tumour activity of NK cells. However, since the antibodies used in many studies were not just selective for NK cells, caution is therefore required in interpreting results obtained from these studies (Vivier *et al*., 2008). Nevertheless, other studies support a role for NK cells in the control of tumour development in mice. Mouse NK cells were involved in the rejection of transplanted tumours and the presence or absence of NK cell receptor ligands on the cell surface of tumour cells made them more susceptible to NK cell-mediated lysis (Stewart and Vivier, 2007). In other experimental studies, activation of NK cells induced subsequent development of T cell responses to tumour cells (Diefenbach *et al.,* 2001). Moreover, blocking of NK cell Ly49 inhibitory receptors in mice increased NK cell activity against tumour cells (Koh *et al.,* 2001). NK cells are also mediators of the anti-tumour effects of several cytokines such as IL-2, IL-12, IL-18 and IL-21 (Stewart and Vivier, 2007).

Clinical and experimental studies confirm an important role for NK cells in human cancers and their therapy. In an 11-year follow up epidemiological survey, it was shown that the extent of NK cell activity in peripheral blood is associated with cancer risk in adults. Thus low NK cell activity is associated with increased cancer risk (Imai *et al.,* 2000). Studies with intratumoral NK cells have faced difficulties because of the low

number of these NK cells and the difficulties in isolating them. However more recent studies have disclosed the phenotypic status and function of NK cells in tumour site and in peripheral blood (Levy *et al.,* 2011). The presence of NK cells in the tumour site indicated a good prognostic factor against different carcinomas (Coca *et al.,* 1997, Ishigami *et al.,* 2000 and Villegas *et al.,* 2002). All the evidence supporting the role of NK cells in tumour control encouraged the application of NK cell-based immunotherpautic strategies. These approaches could be generally summarised into two main categories (Levy *et al.,* 2011) 1) allogeneic or autologous transfer of NK cells (Ruggeri *et al.,* 2007), 2) infusion of tumour-specific monoclonal antibodies that trigger antibody-dependent cytotoxicity by NK cell cytotoxicity and IFN-γ secretion (Terunuma *et al.,* 2008). Both approaches can be improved with co-administration of cytokines that enhance NK cell expansion and function. Thus NK cells play an important role in immunity against tumours and provide a potential tool for cancer therapy.

4.1.12.2 NK cells in human autoimmunity

Autoimmune diseases commence in steps, including release of self-antigens from the target organ, a priming step in secondary lymphoid organs and immune cells homing to the target organ/tissue and subsequent tissue damage and NK cells can act at all these stages (Flodstron-Tullberg *et al.,* 2009). Autoimmune diseases with the involvement of NK cells include: multiple sclerosis (MS), rheumatoid arthritis (RA), systemic lupus arthritis, and type I diabetes mellitus or in conditions associated with autoimmune diseases such as macrophage activation syndrome (MAS).

In MAS, NK cell function was found to be absent or depressed with significant decrease of NK cell cytotoxicity, and this resulted in increased T cell and sustained macrophages activation and the production of large quantities of proinflammatory cytokines (Ravelli,

2002). NK cells can also accumulate in certain inflammatory lesions and in the presence of the appropriate cytokine environment can engage with monocytes to amplify the inflammatory response. Rheumatoid arthritis (RA) represents a good model of this chronic inflammatory process (Dableth *et al.,* 2004). Patients with MS were found to have fewer blood NK cells compared with healthy individuals and NK cells isolated from those patients were found to be impaired in effector function (Vranes *et al.,* 1989). Supporting a protective role for NK cells, MS patients who were in remission stage had high frequencies of $CD95^{+}$ (Fas) cells in their blood. These cells are distinguished by the production of T_H2 cytokines and can actively suppress the pathogenic T cells that can enhance inflammatory responses in the CNS (Takahashi *et al.,* 2001). Both reduction in numbers and functions of NK cells were described in studies with patients with type I diabetes (Flodstron-Tullberg *et al.,* 2009). Moreover, some individuals with naturally occurring mutations in genes that cause defects in NK cells typically suffer chronic infections in childhood and autoimmune manifestations later in life (Monis-Teisserence *et al.,* 1999).

4.1.12.3 NK cells in pregnancy

Enrichment of natural killer cells is observed in the pregnant endometrial tissue in many species. These cells represent a distinct subset of NK cells that are able to secrete pro-angiogenic factors such as vascular endothelial growth factor and placental growth factor (Hanna *et al.,* 2006). They play a role in the remodelling of the arterial system that support maternal endometrial tissue at sites of implantation and help further placental development (Hanna *et al.,* 2006).

4.1.13 The role of NK cells in infection with *Cryptosporidium* species.

The exact role of NK cells in innate immunity to *C. parvum* is unclear but some studies imply these cells may be involved. Human peripheral blood NK cells treated with IL-15 were shown to have cytolytic activity against human intestinal epithelial cell lines infected with *C. parvum* and intestinal expression of this cytokine has been detected in humans (Dann *et al.,* 2005). The same study also proposed that the activation receptor NKG2D was involved in cytotoxicity since its ligand, MICA, had increased expression in an infected human epithelial cell line and also in the intestinal epithelium of infected patients. Also, mice with the *beige* mutation that have NK cells but are deficient in NK cell cytotoxicity (Bannai *et al.*, 2000) developed heavier *C. parvum* infection than mice with normal NK cell functions (Enriquez and Sterling, 1991).

In vitro studies have also examined the activity of NK cells in the presence of sporozoite antigen. SCID mouse splenocytes (lacking T and B cells) produced IFN-γ after stimulation with purified live *C. muris* sporozoites. The production of IFN-γ decreased when NK cells were depleted in the presence of anti-asialo GM1 antibodies and complement in an antibody concentration dependent fashion. Similarly, IFN-γ levels were reduced by treatment with anti-IL-12 and anti-TNF-α neutralizing antibodies, while anti-IL-10 on the other hand increased IFN-γ release (McDonald *et al*., 2000).

The above observations support a role for NK cells in the innate immune response to *C. parvum* but the results of early murine studies could not provide evidence for an *in vivo* protective role for these cells. Treatment of adult SCID mice with anti-asialo GM1 antibodies that depletes NK cells failed to show any effect on the course of infection (Ungar *et al.,* 1991; McDonald and Bancroft, 1994). Further work in this area is

required, therefore, and this project aims to investigate further the role of NK cells in immunity to *C. parvum.*

4.1.14 Hypothesis and aims

As discussed above, NK cells are important in immunity to other intracellular parasitic protozoa. However, although some studies provide indirect evidence for a protective role for NK cells in innate immunity to *C. parvum*, clear unequivocal evidence for involvement of these cells is still lacking. In this Chapter the hypothesis is that NK cells play a central role in the protective innate immune response to *C. parvum*, in part by producing IFN-γ

The aims are therefore:

1. To study *in vitro C. parvum*- mediated activation of IFN-γ by NK cells and regulation of this activity.
2. To investigate splenic NK cell activity in severely infected adult SCID mice.
3. To clarify the protective role of NK cells *in vivo* using gene knockout strains of immunocompromised mice, including one that lacks NK cells.

4.2 MATERIALS AND METHODS

4.2.1 Antibodies

Antibodies to murine IL-12, IL-15, IL-1 and TNF-α were obtained from R&D Systems. The concentrations of antibodies employed were based on results of preliminary experiments with a wide range of concentrations. Titrated sheep anti-IFN-α/β serum was a gift from Dr Michael Tovey (Institute Andre Lwoff, Villegiuit, France). The H22 hamster anti-mouse IFN-γ IgG monoclonal antibody (R&D Systems) in PBS was employed to neutralise IFN-γ. The antibody (100µg in 100µl PBS) or PBS alone (control) was administered ip before oocyst inoculation.

4.2.2 RNA extraction, semi-quantitative and real-time quantitative PCR.

Extraction of RNA from intestinal tissue samples from Rag2$^{-/-}$ and Rag2$^{-/-}$γc$^{-/-}$ mice and reverse transcription to cDNA were performed as described in Chapter 2.

For real-time quantitative PCR, amplification was achieved with 20pmol of each oligonucleotide primer. The primer sequences were as follows: murine β-actin forward CCT TCC TTC TTG GGT ATG GAA T and reverse GCA CTG TGT TGG CAT AGA GGT (106 bp); murine IFN-γ forward GCC AAG TTT GAG GTC AAC AAC and reverse ATC AGC AGC GAC TCC TTT TC (121bp). Reaction mixtures were setup to a final volume of 20µl using a total of 100ng cDNA, 20pmol of each primerand 10µl FastStart SYBR Green master mix.

4.2.3 Purification of NK cells by magnetic activated cell sorting

NK cells were purified by magnetic separation using magnetic microbeads conjugated with an antibody specific for NK cells. A SCID splenocyte suspension was prepared as described previously. The cells were centrifuged and resuspended to 1×10^7 in 90µl of buffer (PBS with 0.5% BSA and 2mM EDTA). The cells were incubated for 20 minutes with 10µl of CD49b (DX5) magnetised microbeads (Miltenyi Biotec) at 4°C, then washed in 1-2ml of buffer, and re-suspended in 1ml of buffer. A miniature magnetic activation cell sorting (MACS) column (Miltenyi Biotec) was initially stabilized at the MACS separator, then rinsed with 3ml buffer. The cell suspension was then passed through the column. Unlabeled cells passing through were collected and remaining unlabelled cells obtained after the column was washed 3 times with 3mls of buffer. To collect CD49b-labelled cells, the column was detached from the separator, filled with 5ml of buffer and immediately flushed out with the supplied plunger.

4.3 RESULTS

4.3.1 *In Vitro* splenic NK cell production of IFN-γ induced by *C. parvum* antigen

4.3.1.1 Stimulation of IFN-γ production by SCID splenocytes cultured with *C. parvum* oocysts

The first study was designed to investigate if *C. parvum* oocysts would stimulate SCID mouse splenocytes to produce IFN-γ. The cells were seeded in different numbers (1×10^5, 2×10^5 or 4×10^5) into 96-well plates and were either co-cultured with 2×10^5 oocysts or had no parasite stimulation. Supernatants were collected after 48h incubation at 37^{o}C and production of IFN-γ was measured by ELISA. As shown in Fig 31, low levels of IFN-γ were detected in supernatants from unstimulated cells or from cell numbers up to $2X10^5$, but a significant amount of cytokine was produced in cultures with 4×10^5 cells ($p<0.0032$). This result suggests that $4X10^5$ cells is sufficient for stimulation of IFN-γ production by *C. parvum* antigen. This number of cells was used in subsequent experiments.

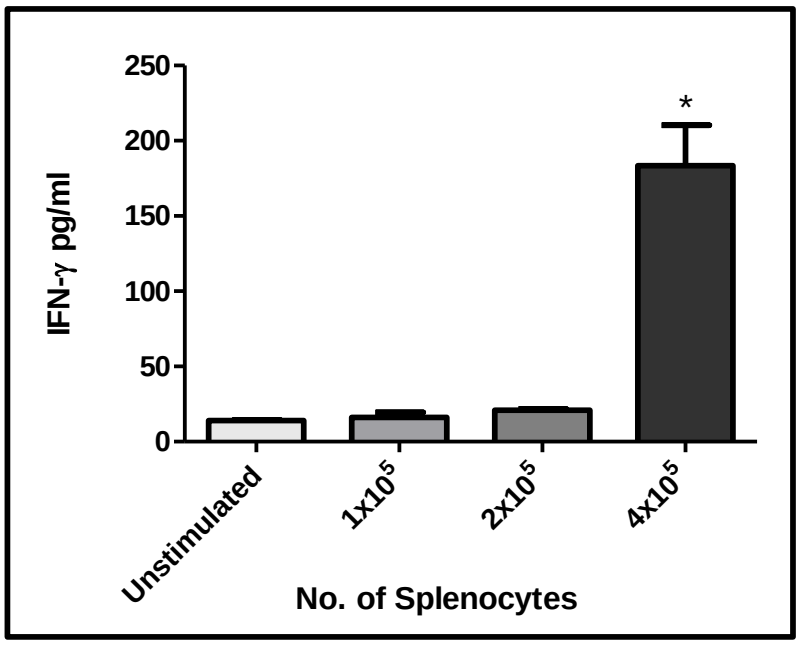

Figure 31.ELISA measurement of IFN-γ production by SCID splenocytes following stimulation with *C. parvum* oocysts. A significant level of IFN-γ was detected with $4x10^5$ cells (*p <0.0032).

4.3.1.2 The effect of different numbers of *C. parvum* oocysts on the release of IFN-γ from splenocytes.

To establish an optimal number of oocysts that would stimulate SCID mouse splenocytes to produce IFN-γ, 4×10^5 cells in a 96 well plate were stimulated with different numbers of oocysts. The level of the cytokine in the supernatants was then measured. As shown in Fig 32, all oocyst numbers stimulated significant IFN-γ expression (with a significance of p<0.04 observed with the highest number of oocysts) and the highest amount occurring with 1-2×10^5oocysts ($2x10^5$ oocysts: p<0.0057). $2X10^5$ oocysts was therefore used in all the followingexperiments that involved *C. parvum* stimulation of cultured splenocytes. In cultures with $2X10^5$ oocysts, IFN-γ

expressions was significantly greater than in cells stimulated with $1x10^6$ oocysts (p =0.03).

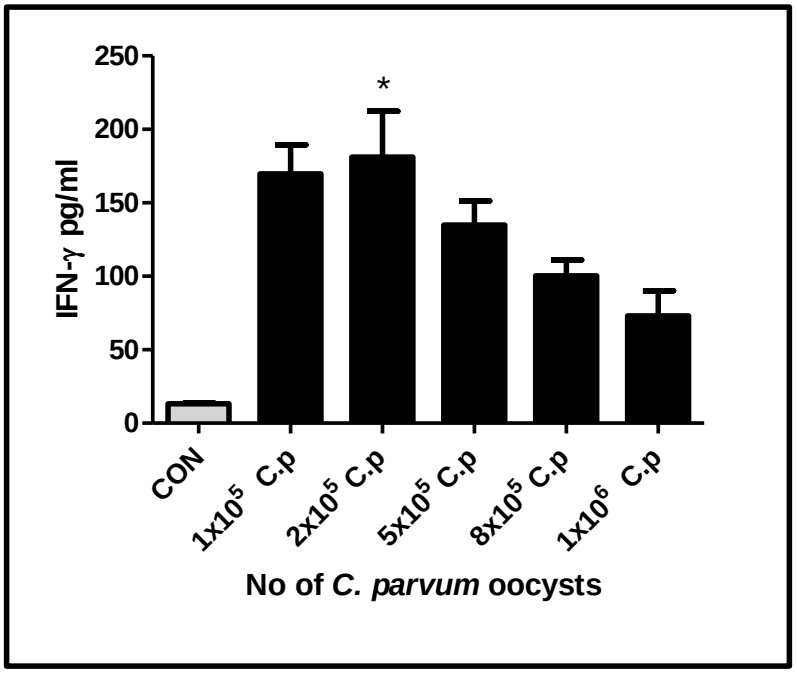

Figure 32.The effect of different numbers of *C. parvum* oocysts on IFN-γ production in cultured SCID splenocytes as measured by ELISA. All numbers of oocysts were able to stimulate the cells to produce IFN-γ. The highest cytokine expression was observed with $2X10^5$ oocysts (*p<0.0057).

4.3.1.3 Comparison of the effect of *C. parvum* oocysts and sporozoites on splenocyte stimulation

To determine if sporozoites were important in stimulating IFN-γ, a comparison was made of the ability of purified sporozoites and oocysts to induce cytokine production. In addition, whether live sporozoites provided better stimulation than killed parasites was investigated. Sporozoites were excysted from oocysts by incubation with the bile salt sodium deoxycholate in a concentration of 0.1% wt/volume for 90 minutes at 37°C. Excysted sporozoites were purified from oocysts and oocyst shell debris by passing through a 5 µm diameter pore filter. Microscopic observation confirmed that sporozoites were completely free of oocysts and empty oocyst shells. The sporozoites were then washed with culture media, resuspended and counted microscopically by using a haemocytometer. To obtain killed parasites, sporozoites were frozen at -80^{O}C for 3 hours.

SCID mouse splenocytes were cultured as before and stimulated with either 2x10^{5} oocysts or 8x10^{5} live or freeze-thawed sporozoites as an equivalent (4 sporozoites in an oocyst). Similar high amounts of IFN-γ were detected in from the different *C. parvum* preparations but not from control samples (Fig 33). These results therefore indicated that the sporozoite and not the oocyst shell is the predominant stimulant for IFN-γ production by cultured splenocytes and that the sporozoites need not be alive to provide a strong antigenic stimulus.

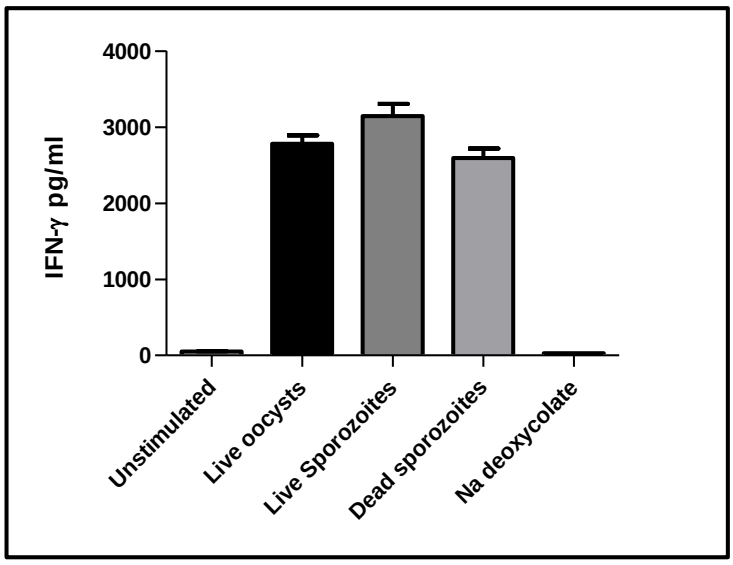

Figure 33. IFN-γ levels as measured by ELISA in cultured splenocytes stimulated with either *C. parvum* oocysts, live or dead sporozoites. Splenocytes were stimulated with live oocysts or the equivalent number of live/ dead sporozoites and supernatants were tested for IFN-γ release. No differences in IFN-γ levels were detected from the different antigen stimulated samples ($p<0.13$, $p=0.11$).

4.3.1.4 The effect of treatment with different cytokines on IFN-γ production by SCID mouse splenocytes

Many cytokines activate NK cells to produce IFN-γ including IL-15, IL-12, IL-18, IL-1, TNF-α, IL-10 and type IFNs (Lieberman and Hunter2002; Zucchini *et al.,* 2008). Therefore, the following experiments studied the role of various proinflammatory cytokines in stimulating NK cells. This was done by employing in the cell-oocyst culture system neutralising antibodies for individual cytokines.

4.3.1.4.1 The effect of anti-IL-12

Studies with other models of infections have indicated that IL-12, a major product of DCs, is a central cytokine in NK cell activation to produce IFN-γ in response to infection

(Lieberman and Hunter2002). It has also been established that IL-12 is a major stimulus for IFN-γ production in neonatal SCID mice infected with *C. parvum* (Urban *et al.*, 1996). Therefore it was necessary to investigate the role of this cytokine on NK cell activation by *C. parvumin vitro*. SCID mouse splenocytes were stimulated with *C. parvum* oocysts and cultured with or without different concentrations of anti-IL-12 neutralising antibody. Fig 34 demonstrates a significant inhibition of IFN-γ secretion in antibody treated cultures in comparison to untreated cells and this effect was dose-dependent (p<0.007).

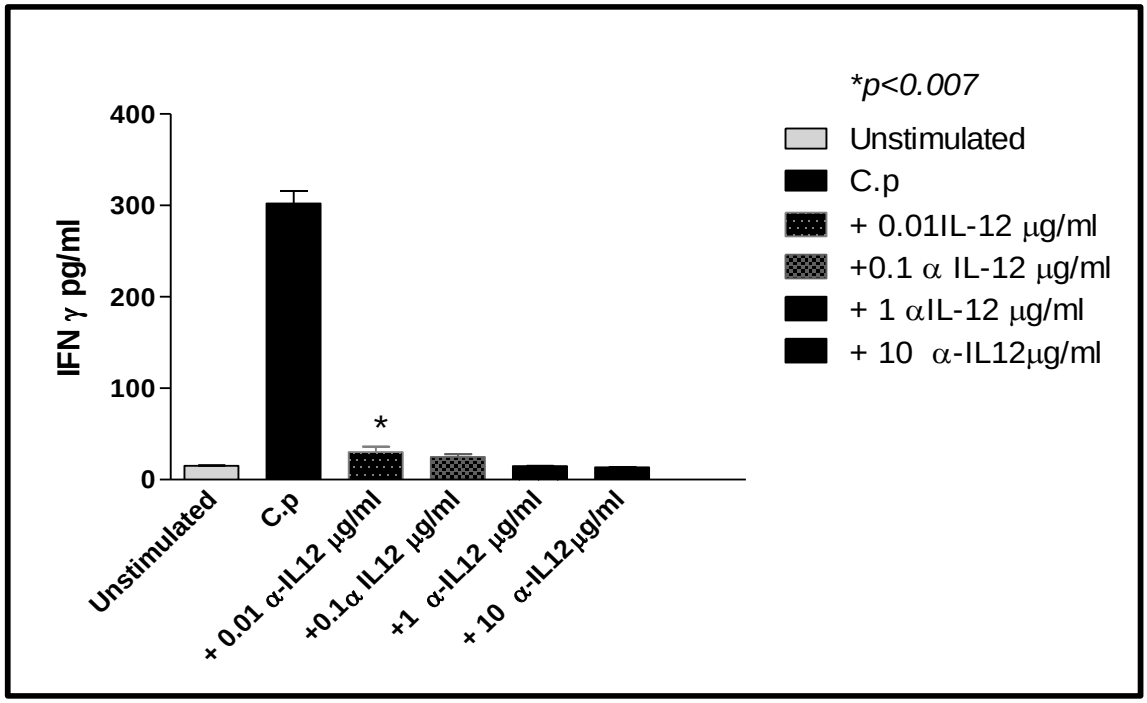

Figure 34.The effect of anti- IL-12 antibody on IFN-γ production by SCID splenocytes as measured by ELISA. Cultured splenocytes were cultured with oocysts alone or with different concentrations of anti–IL-12 antibody and their ability to release IFN-γ was tested by ELISA.

4.3.1.4.2. The effect of anti-TNF α, anti- IL-15 and anti-IL-1

Some other cytokines known to be involved in NK cell activation were examined. Spleen cells were stimulated with *C. parvum* antigen and incubated with three different concentrations of neutralising antibodies to TNF-α, IL-15 or IL-1. Positive controls were cultured with the oocysts only. At 48h postinfection IFN-γ production in the supernatants was measured by ELISA. Confirming previous results a significant level of IFN-γ was measured from supernatants of antigen stimulated cells. This effect was significantly reduced with all antibody-treated cells and was slightly dose-dependent. The strongest inhibitory effect was observed in cells treated with anti-TNF-α (p=0.0007) while the weakest effect was with anti-IL-1 (p=0.02). Fig. 35 illustrates these results.

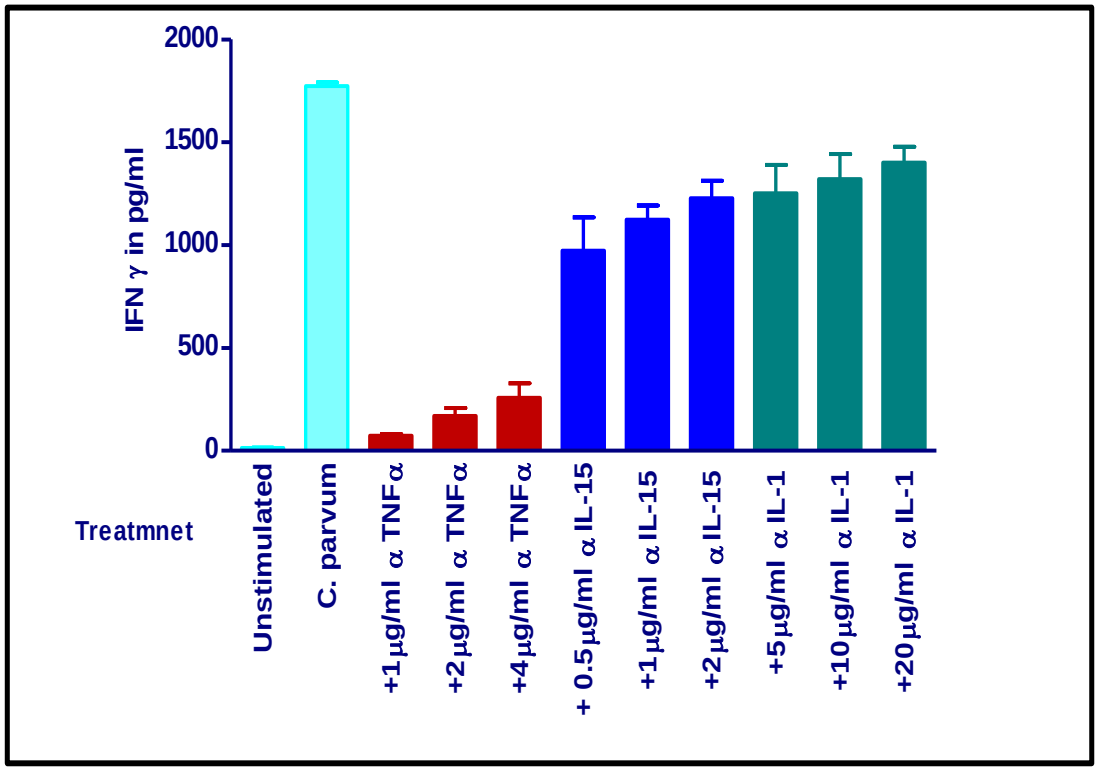

Figure 35 .The effect of treatment with different cytokine neutralising antibodies on IFN-γ production by SCID mouse splenocytes. Cultured splenocytes were stimulated with an antigen in the presence or absence of different concentrations of antibodies to either TNF-α, IL-15 or IL-1 and IFN-γ was measured by ELISA. Treatment with antibodies significantly reduced IFN-γ expression particularly in cells treated with anti-TNF-α (*p*=0.0007*).*

4.3.1.4.3 The effect of anti-type I IFN on IFN-γ production

Previous studies with other infection models indicated that type I IFNs produced by DCs can stimulate NK cells directly or indirectly (Zucchini *et al.,* 2008). Also, as indicated in Chapter 3 of this thesis, type I IFNs play a protective role in innate immunity to *C. parvum.* Hence, it was important to study the effect of type I IFN on splenocyte stimulation by *C. parvum* oocysts. This was done using a sheep anti-mouse type I IFN serum that has been used extensively by many groups. There was a

significant decline of IFN-γ expression in supernatants from antibody-treated cells in comparison to the control samples and this was dose dependant (Fig. 36; $p<0.00052$).

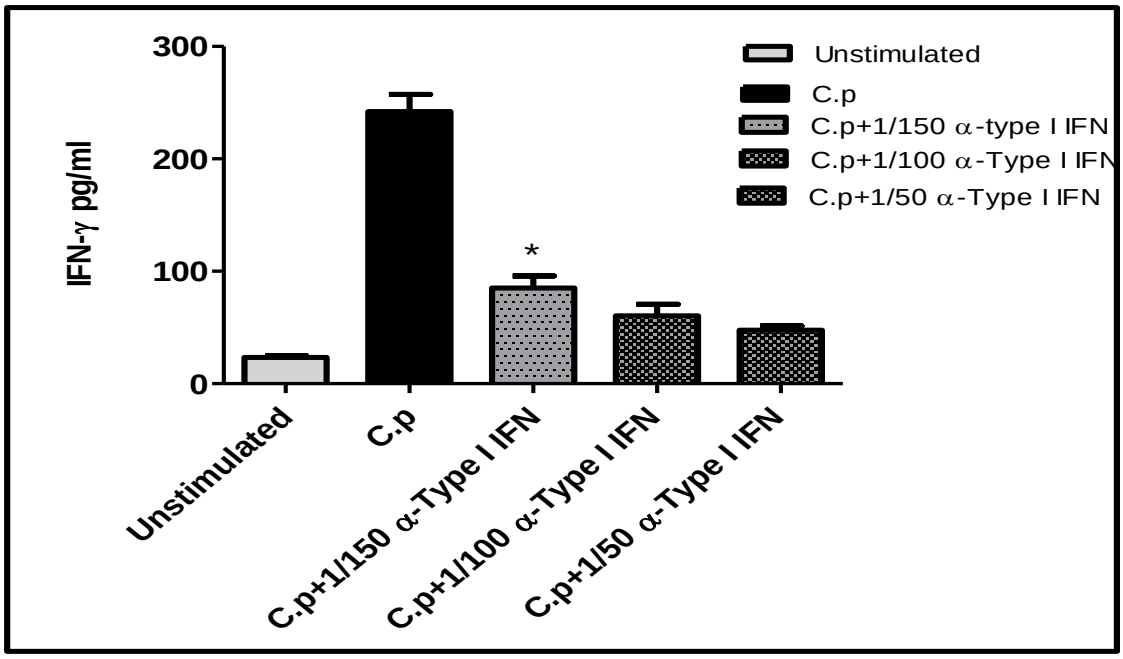

Figure 36The effect of treatment with anti-type I IFN on IFN-γ production by SCID splenocytes measured by ELISA. Treatment with anti-type I IFN decreased IFN-γ production in a dose-dependent matter (*$p<0.00052$).

The results of the previous three experiments indicate that IL-1, IL-12, IL-15, TNF-α and type I IFN stimulate IFN-γ production in SCID mouse splenocytes after stimulation with *C. parvum.*

4.3.1.5 The effect of anti-type I IFNs on IL-12 production by SCID splenocytes

As well as IL-12, DCs are a major source of type I IFNs that stimulate DCs further and also activate NK cells (Zucchini *et al.,* 2008). An examination was made therefore of the effect of type I activity on IL-12 expression during stimulation of SCID mice splenocytes with oocysts. Cultured splenocytes were stimulated with oocysts in the presence or absence of anti-type I IFN serum (diluted 1/50 from the $3.2X10^5$ U/ml of original material). The supernatants were collected after 24 h and IL-12 level was measured by ELISA.

Fig 37 demonstrates there was significant expression of IL-12 in response to *C. parvum* stimulation ($p<0.03$) and this was decreased by treatment with anti-type I IFN antibody ($p<0.0001$). These results suggest that type I IFN directly or indirectly enhances production of IL-12 by splenocytes in response to *C. parvum*.

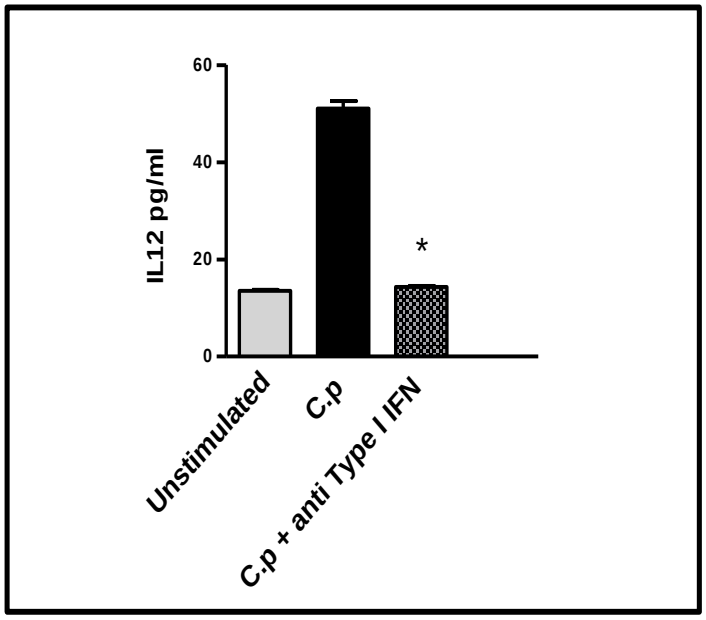

Figure 37.ELISA measurement of IL-12 production by SCID mouse splenocytes in the presence of *C. parvum* antigen alone or with anti-type 1 IFN antibody. IL-12 production by splenocytes was inhibited by the treatment with anti-type 1 IFN antibody (*$p<0.0001$)

4.3.1.6 NK cells in SCID mice splenocytes as a source of IFN-γ

The previous results indicated that in response to *C. parvum* stimulation, culture splenocytes produced significant amount of IFN-γ through the activity of different cytokines and as NK cells represent a major cellular source of IFN-γ, it became paramount to establish if these cells are the source of this cytokine in *C. parvum* stimulated SCID splenocytes.

4.3.1.6.1 Isolation of NK cell enriched and depleted cell populations from SCID splenocytes

NK cells were isolated from SCID mouse spleen cell suspension using CD49b (DX5) coated microbeads as described in Materials and Methods. Cell suspensions from positively and negatively selected cells as well as unfractionated splenocytes were re-suspended in FACS buffer then cultured with anti CD49b antibody and analysed by FACS.

As shown in Fig 38, 5% of the total unsorted cells stained for CD49b, while 80% of the NK cell enriched population were $CD49b^+$ and only 1% of the NK cell depleted population were $CD49b^+$.

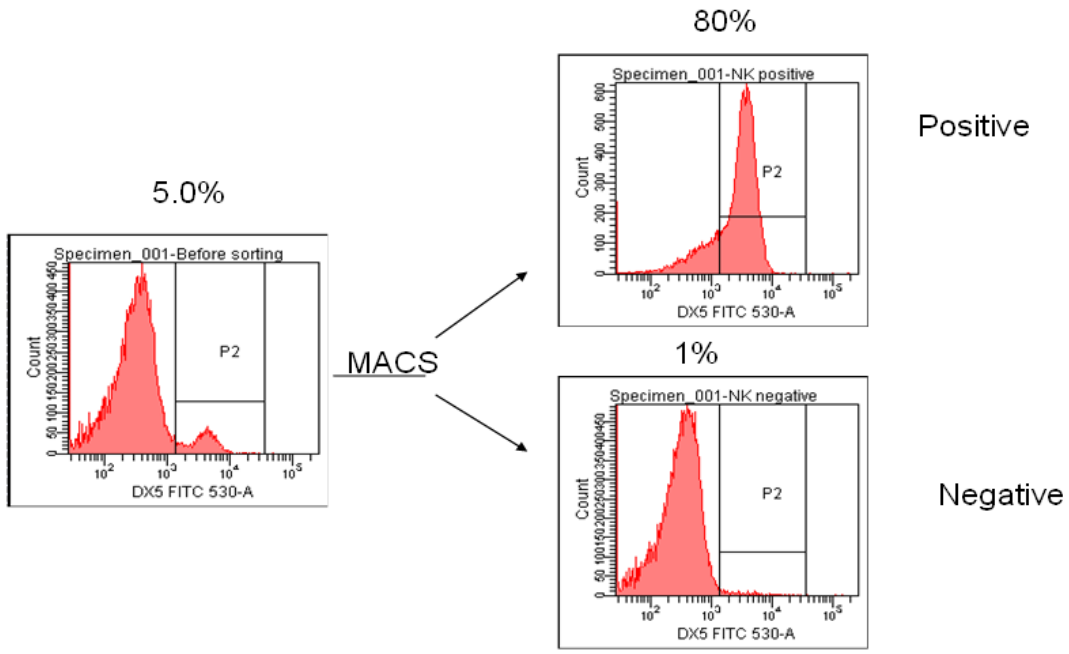

Figure 38. FACS analysis of SCID splenocytes before and after positive isolation of NK cells

4.3.1.6.2 IFN-γ production by NK cell enriched and NK cell depleted SCID mouse splenocytes stimulated with *C. parvum* oocysts

The NK cell enriched and depleted populations plus original cell population were seeded in a 96-well plate and stimulated with oocysts. As shown in Fig 39, no IFN-γ was detected in supernatants of negatively selected cells depleted of NK cells whereas IFN-γ was detected in cultures with unfractionated and NK cell enriched populations stimulated with antigen (*p<0.012). A significantly higher amount of cytokine was obtained with the NK cell enriched population, however (**p<0.013).

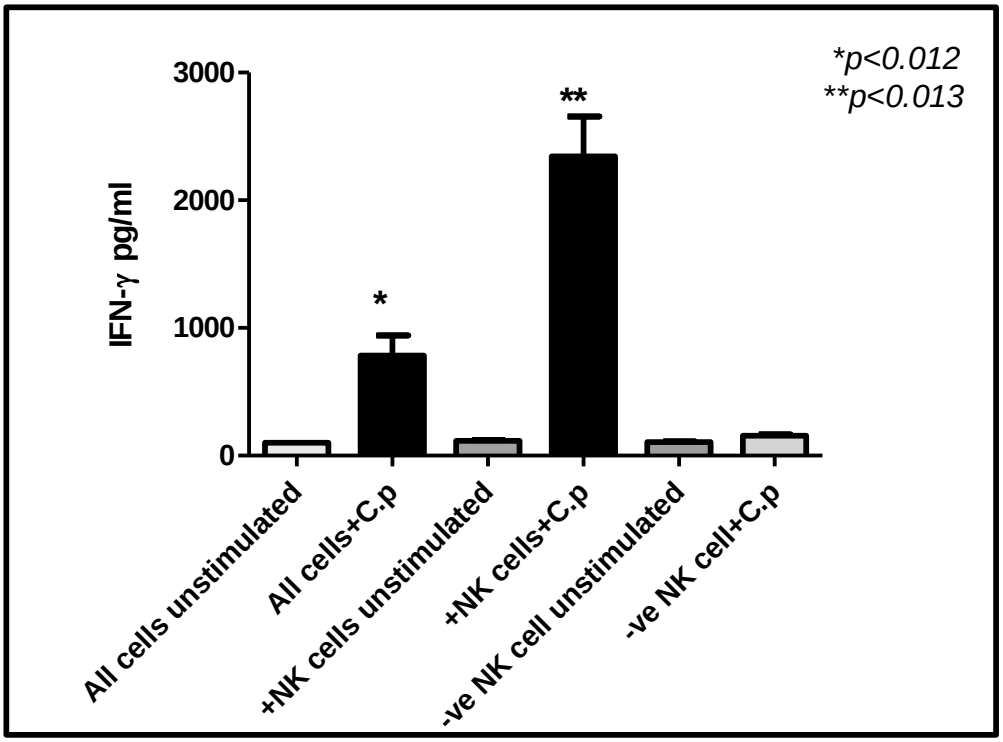

Figure 39. ELISA measurement of IFN-γ production by NK cell enriched or depleted splenocyte populations after stimulation with *C. parvum* antigen. NK cells were purified by MACS and IFN-γ production by this population was compared with that of the NK cell depleted and or original populations.

4.3.1.6.3 The effect of *C. parvum* stimulation on type I IFN production by macrophages and dendritic cells

As it was already shown treatment of splenocytes with antibody to type I IFN decreases their ability to produce IL-12, it was interesting to see if splenocytes culture depleted of NK cells that are assumed to contain macrophages and DCs can produce type I IFN in response to stimulation with *C*. *parvum*.

Splenocytes depleted of NK cells by MACS using CD49b (DX5) MicroBeads. were stimulated with *C. parvum* oocysts or cultured with medium only for the negative control. After incubation period of 24h supernatants were collected to test the release of

IFN–α using a bioassay of anti viral activity employing the murine fibroblastic cell line L929 infected with EMCV (method discussed earlier in Chapter 3). As illustrated in Fig 40, supernatants from oocysts stimulated cells, showed a significant increase (p<0.01) of antiviral activity in comparison to the unstimulated cells indicating the production of type I IFN by cells depleted of NK cells.

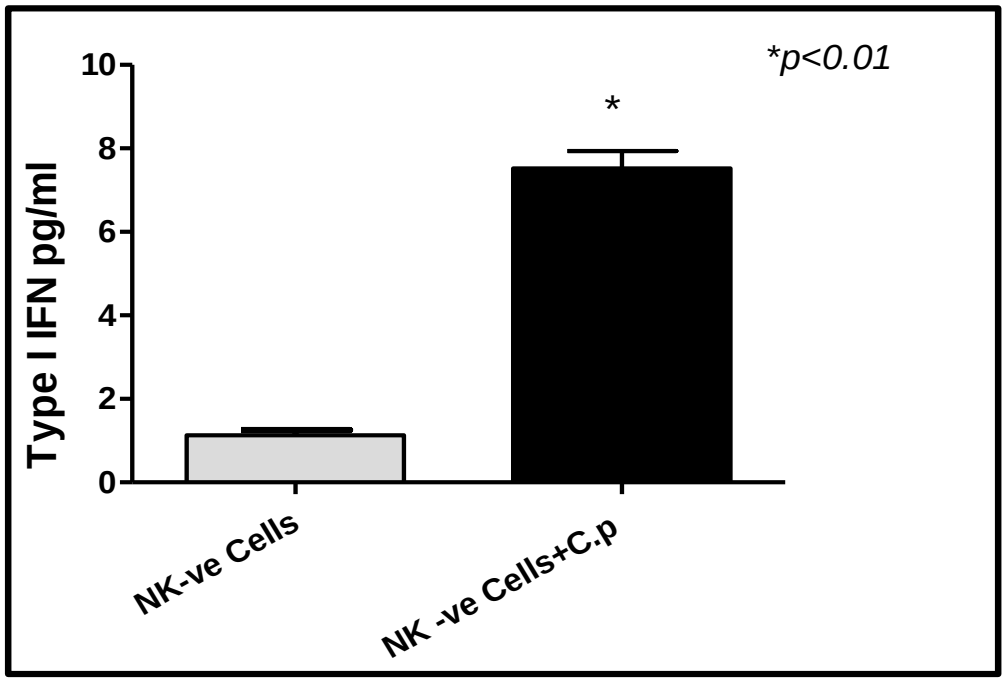

Figure 40.Antiviral assay to measure type I IFNs production by cultured splenocytes lacking NK cells. Splenocytes were depleted of NK cells by MACS, stimulated with *C. parvum* and type IFN I production measured in the supernatants using a bioassay with standards of IFN-α4.

4.3.2 Splenic NK cell activity in chronic infection of SCID mice

Previous work in this lab studied chronic infection of adult BALB/c SCID mice with *C. parvum*. Results indicated that infection of adult SCID mice increased only gradually and became fulminant and caused death by about 3 months after infection (McDonald and Bancroft, 1994). Interestingly, when mice were infected as neonates there was an early acute phase of infection at the peak of which the animals produced many oocysts. After recovery, a similar pattern of infection to that described in adults was obtained (Fig 41).

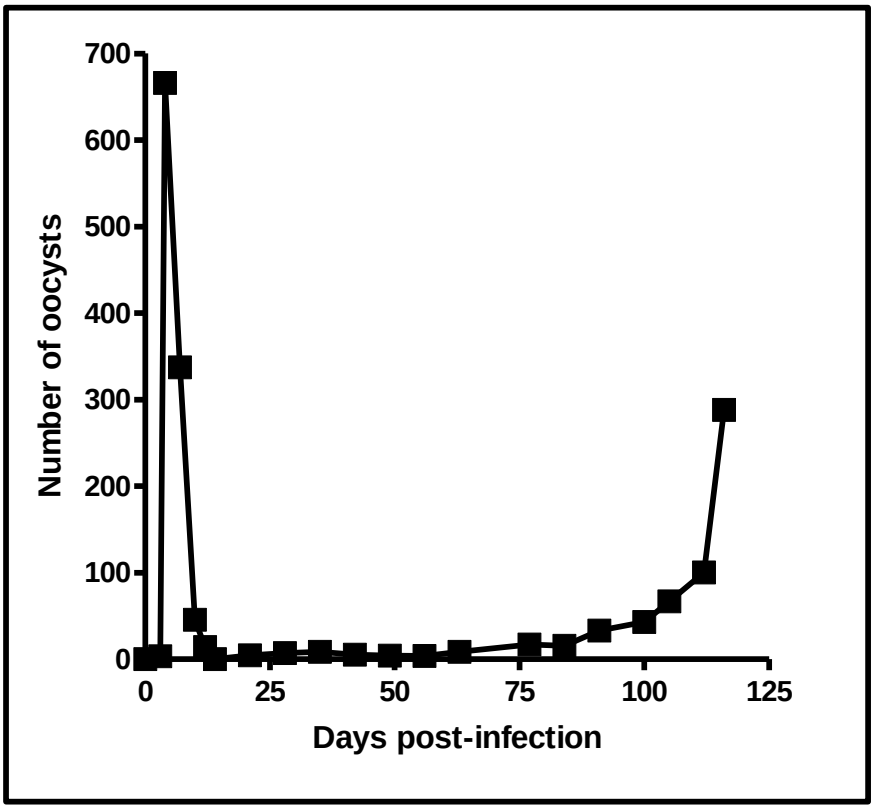

Figure 41. Pattern of chronic *C. parvum* infection in SCID mice infected as neonates. An early acute phase was followed by low level of infection for several weeks, then the final fulminant phase occurs with most mice dying.

The long term survival of SCID mice is dependent on IFN-γ (McDonald and Bancroft, 1994). Therefore experiments were designed to examine NK cell activity in chronically infected mice that were shedding large numbers of oocysts and showing morbidity.

4.3.2.1 Chronic *C. parvum* infection of adult SCID mice

Six weeks old SCID mice were infected with 1×10^6 oocysts by oral gavage. Stool samples were collected weekly and the level of infection was assessed by counting parasites microscopically. The level of infection initially was very low but after about 100 days postinfection mice were starting to show signs of morbidity and would eventually die. Signs of illness included: piloerection, weight loss, soft stool and jaundice seen in ears (and sometimes paws).

At this stage the number of parasites in stool samples were usually found to be relatively high (>70 oocysts/50 high powered fields). Some animals that looked jaundiced, however, shed few oocysts but histopathological examination of hepatic tissue samples confirmed a hepatic involvement as a high number of oocysts was detected in the bile ducts (Fig 42).

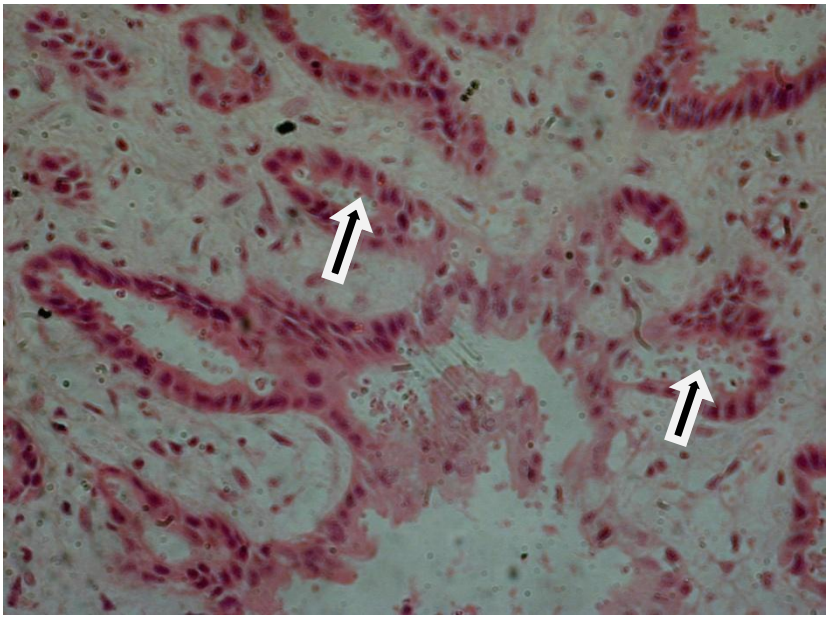

Figure 42 Hepatic duct *C. parvum* infection of a SCID mouse.
Chronically infected SCID mouse looked jaundiced and hematoxylin/eosin stained liver sample confirmed hepatic bile duct infection (arrows).

4.3.2.2 IFN-γ production by splenocytes of severely infected mice

Pooled splenocytes from severely infected mice or mildly infected mice (<10 oocysts/50 fields) or from healthy uninfected control mice of the same age were cultured in 96-well plates with or without stimulation with *C. parvum* oocysts. Culture supernatants were collected after 48 h and the production of IFN-γ was measured by ELISA.

Fig 43 shows that after stimulation with the parasite antigen there was no increase in IFN-γ in supernatants of highly infected mice in comparison to a significant increase in cytokine level in supernatants obtained from splenocytes of uninfected animals (*p<0.007). Interestingly, a higher concentration of IFN-γ was measured in supernatants from cells of mildly infected mice compared with uninfected mice (**p<0.0006).

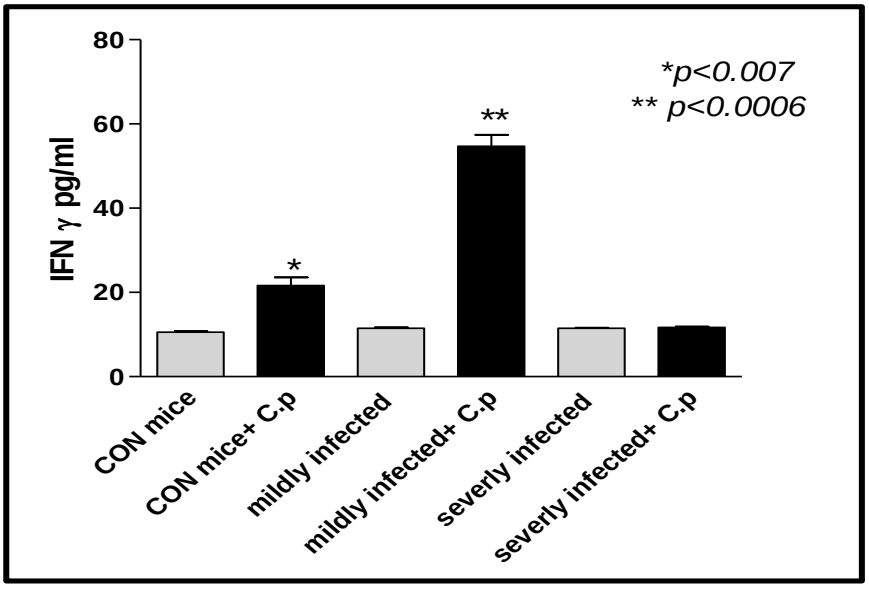

Figure 43. ELISA comparing IFN-γ levels in *C. parvum* stimulated splenocytes at different stages of the infection of SCID mice. When mice showed signs of severe disease, splenocytes were obtained and cytokine production was compared to that of cells from mildly infected or uninfected mice.

4.3.2.3 IFN-γ production by splenocytes of mice with known hepatic infection

Splenocytes from SCID mice with hepatic involvement but were shedding few oocysts were also tested for their ability to be stimulated for IFN-γ production by oocysts. As in the previous section there was no increase in antigen-stimulated IFN-γ production from cells of the hepatically infected mice. However, significant levels of the cytokine were measured in supernatants from antigen stimulated cells obtained from uninfecetd mice (p<0.0004) (Fig 44).

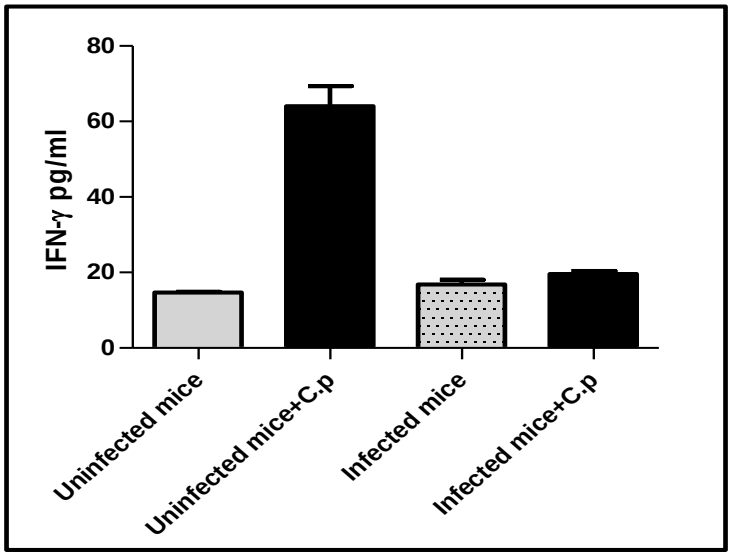

Figure 44. Measurement of IFN-γ levels in cultured splenocytes of mice with *C. parvum* infection of the hepatic system. Antigen did not increase IFN-γ production by cells from mice with hepatic infection.

4.3.2.4 Analysis of the NK cell population in splenocytes of chronically infected mice

The previous results indicated that cultured splenocytes in SCID mice with chronic infection at the fulminant stage lose their ability to produce IFN-γ production in response to antigen stimulation. In the present *in vitro* study NK cells were shown to be a major cellular source of this cytokine. Hence it is it is possible that during severe infection there was a loss of NK cells or a defect in the ability of NK cells to produce IFN-γ. Therefore it was essential to analyse the content of NK cells in SCID mouse splenocytes at the late stage of infection.

Splenocytes from infected mice showing morbidity and shedding many oocysts and from uninfected control mice were incubated with FITC-conjugated anti-CD49b as described in the Materials and Methods and prepared for FACS analysis.

Interestingly, in comparison to splenocytes from uninfected animals that had 11.4% $CD49b^+$ cells, no cells of this type were found in splenocytes of heavily infected mice (Fig 45). This suggested an absence of NK cells in spleens of strongly infected mice which may explain the failure of production of IFN-γ in the spleens of ill mice. Alternatively, infection causes loss of CD49b expression accompanied by loss of ability to express IFN-γ.

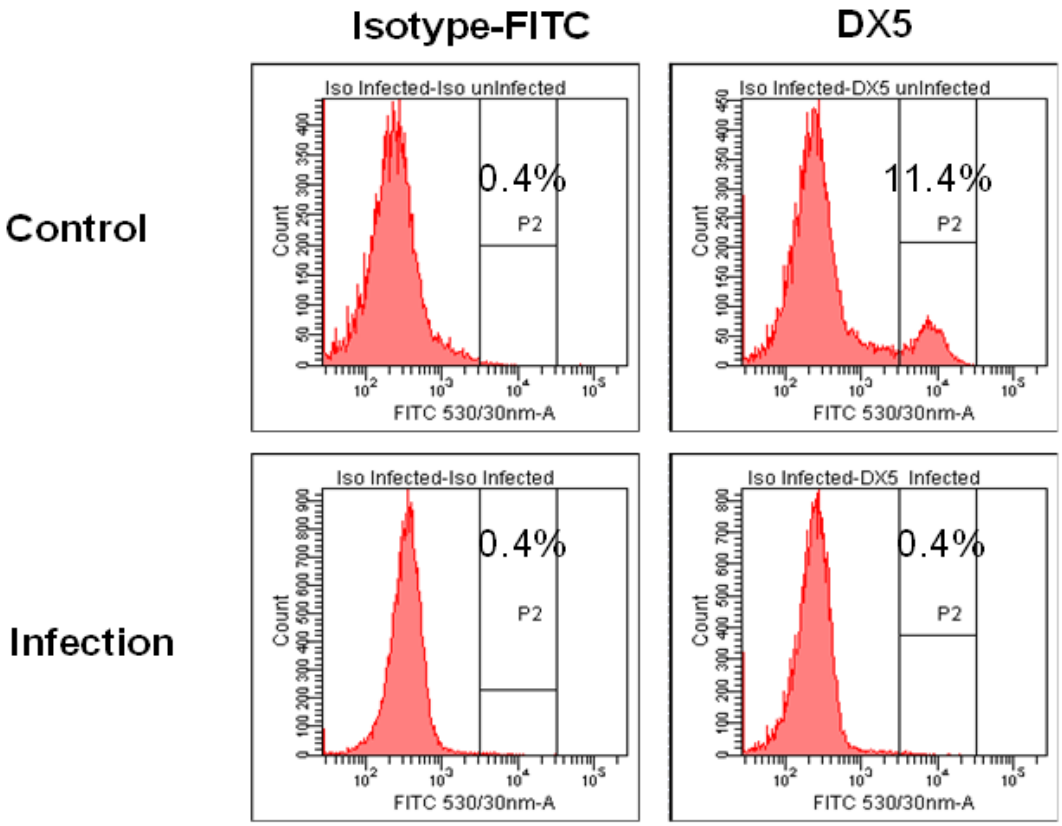

Figure 45.FACS analysis of NK cell population in splenocytes of uninfected and severely infected SCID mice.

4.3.3 *In vivo* studies to investigate the role of NK cells in immunity to *C. parvum*

As discussed earlier in this thesis, previous *in vivo* investigations suggested indirectly that NK cells could play a protective role in innate immunity to *Cryptosporidium* (McDonald *et al.,* 2000; Ungar *et al.,* 1991), but there has been no direct evidence that they are an essential component.In this section therole of NK cells and IFN-γ in the innate responses to *C. parvum* infection is further studied. This was achieved using adult and neonatal mice of the C57BL/6 $Rag2^{-/-}$ strain that lack T and B cells but have normal NK cells and C57BL/6 $Rag2^{-/-}\gamma c^{-/-}$ that, in addition, lack NK cells due to the absence of the common γc chain component of IL-15R (as well as some other cytokine receptors, such as IL-2 and IL-7) and so have no IL-15 function that is essential for NK cell development in mice (Di Santo, 2006). Indeed, these mice have no lymphocytes so their immune response is highly dependent on myeloid cells.

4.3.3.1 *C. parvum* infection of adult $Rag2^{-/-}$ and $Rag2^{-/-}\gamma c^{-/-}$ mice

Weaned (age 4-5 weeks) $Rag2^{-/-}$ and $Rag2^{-/-}\gamma c^{-/-}$ mice were infected with 1×10^6 *C. parvum* oocysts. The infection was measured by counting parasites in acid-fast stained stool smears. Initially, the level of infection was followed daily and then it was checked every week. During the first week of infection, oocyst production was observed in both mouse strains and at the peak this was significantly higher in $Rag2^{-/-}\gamma c^{-/-}$ (*p<0.003). (In older mice this initial acute phase of infection was sometimes absent).

Following the initial acute infection, both mouse strains had a period of remission followed by relapse with a gradual increase in the numbers of oocysts being shed. In the case of the $Rag2^{-/-}\gamma c^{-/-}$ mice, the remission period was shorter and, importantly, by day 60 the infection became more progressive and the numbers of oocysts shed were

significantly higher than in Rag2$^{-/-}$ mice (**p<0.02). Also, from day 91 onward, Rag2$^{-/-}$γc$^{-/-}$ mice started to show signs of morbidity.

On the other hand, the Rag2$^{-/-}$ micehad a longer period of remission and when the infection redeveloped in this strain, the numbers of oocysts shed remained significantly lower when compared to the Rag2$^{-/-}$γc$^{-/-}$ mice. By day 140 post infection, all 6 Rag2$^{-/-}$γc$^{-/-}$ mice were dead while all Rag2$^{-/-}$ mice still looked healthy. Results are summarised in Fig 46 and 47. These observations suggest NK cells are important in innate immunity against *C. parvum* in adult mice.

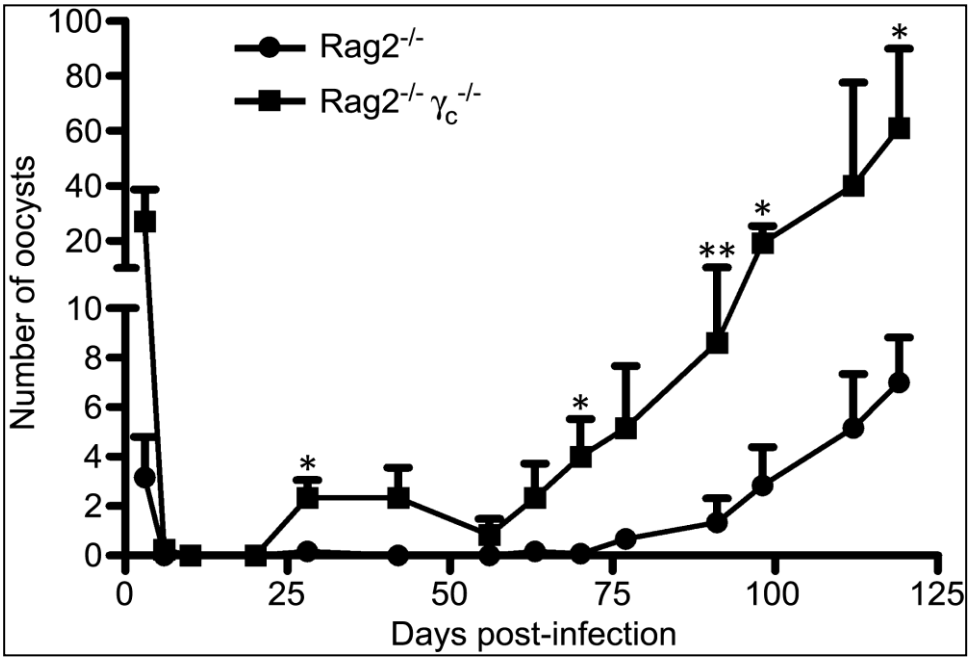

Figure 46.Trend of *C. parvum* infection in adult Rag2-/- and Rag2-/- γc.mice

Adult Rag2$^{-/-}$ and Rag2$^{-/-}$γc$^{-/-}$ animals were infected by *C. parvum* oocysts and microscopic measurement was made of oocyst shedding in acid-fast stained fecal smears from mice at different times of infection. As compared to Rag2$^{-/-}$mice; Rag2$^{-/-}$γc$^{-/-}$ mice showed significantly higher oocyst production in both the acute and the chronic phase of infection(*p<0.003; and **p<0.02 respectively).

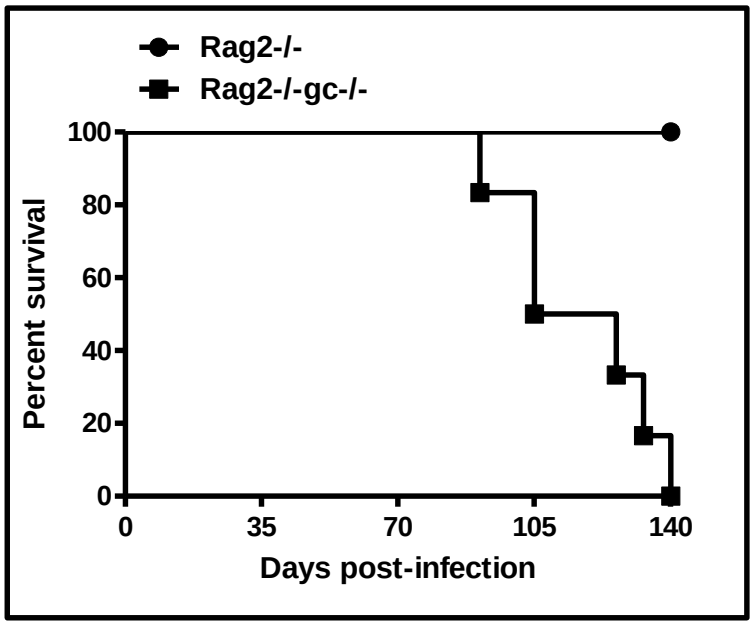

Figure 47.Comparison of survival of adult Rag2-/- and Rag2-/-γc-/-mice following infection with *C. parvum*. From about 90 day postinfection and onward the $Rag2^{-/-}\gamma c^{-/-}$ mice showed mortalities and by day 140 all mice of this group were dead. All the $Rag2^{-/-}$ were still alive at that time.

4.3.3.2 Histological examination of infected colon of Rag2-/-γc-/- mice

To confirm that infection was causing morbidity, colonic tissue samples were obtained from $Rag2^{-/-}\gamma c^{-/-}$mice at different times postinfection and examined histologically (method described in Chapter 2). When the animals looked healthy and few oocysts (around 5 per 50 fields) were detected in faecal smears, no parasites were observed in the crypts and there were no signs of pathology in the colonic sections (Fig 48). However, when the mice showed signs of morbidity accompanied by high numbers of parasites shed in stool, many crypts were infected with large numbers of parasites. Degeneration of the epithelial cells as an outcome of severe infection was also observed.

Low degree of infection **High degree of infection**

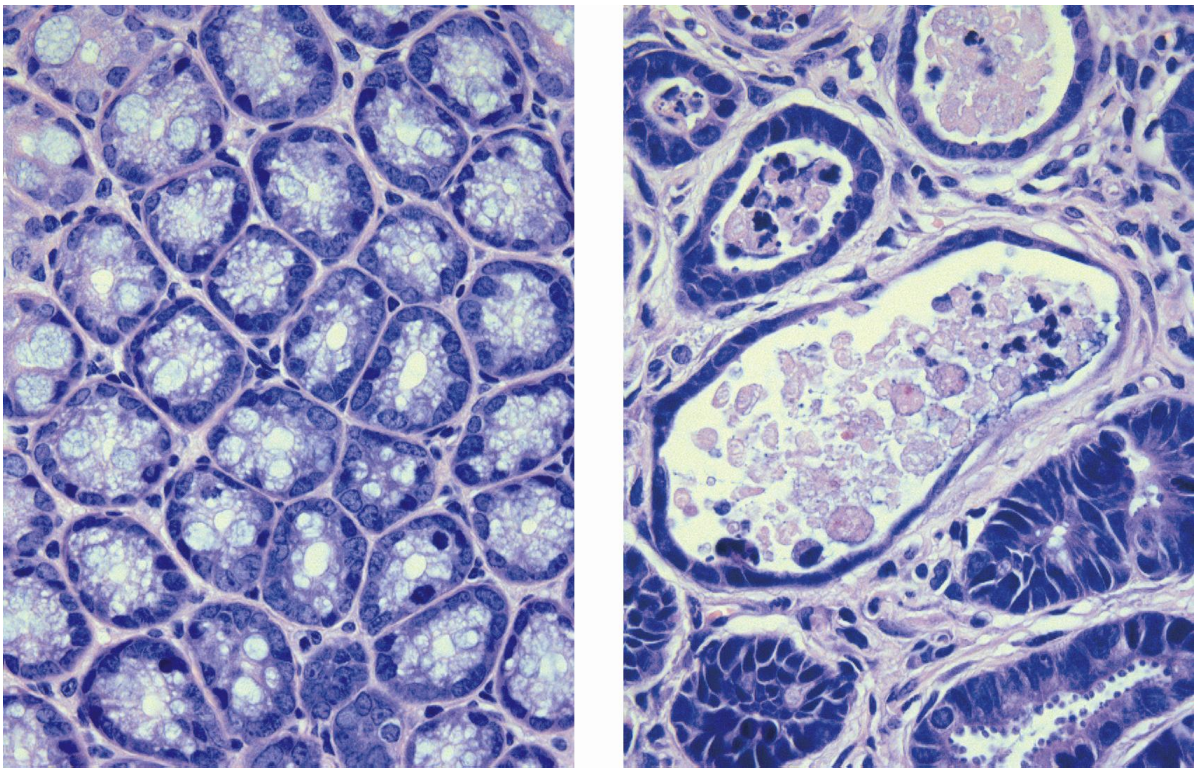

Figure 48. Sections of hematoxylin/eosin stained colon of infected adult Rag2-/-γc-/- mice taken during the middle (excreting few oocysts) and late stages of infection (severe infection). With a low degree of oocyst shedding no parasites could be observed in the crypts and there was no sign of pathology. A high degree of oocyst shedding accompanied by morbidity was associated with many crypts infected with large numbers of parasites and focal degeneration of epithelium. Microscopic study was performed at X400 magnification.

4.3.3.3 Measurement of IFN-γ production by splenocytes of Rag $2^{-/-}$ -and Rag$2^{-/-}\gamma c^{-/-}$ mice after *C. parvum* antigen stimulation

The earlier *in vitro* studies with SCID mice in this Chapter showed that IFN-γ was produced largely by NK cells in splenocytes after stimulation with C. *parvum* oocysts. Hence it was of interest to study the production of this cytokine in splenocytes of Rag$2^{-/-}$ $\gamma_c^{-/-}$ mice.

Cultured splenocytes from Rag $2^{-/-}$ mice and Rag$2^{-/-}\gamma_c^{-/-}$ were stimulated with *C. parvum* oocysts for 48h or left unstimulated before measuring IFN-γ by ELISA. IFN-γ was not detected in supernatants from cells of Rag$2^{-/-}\gamma_c^{-/-}$, but a significant amount of cytokine was found in supernatants from cells from Rag $2^{-/-}$ mice when compared to control samples (*p*<0.006*)* (Fig 49*).* These results agree with the earlier finding that SCID splenocytes were unable to produce IFN-γ after depletion of NK cells.

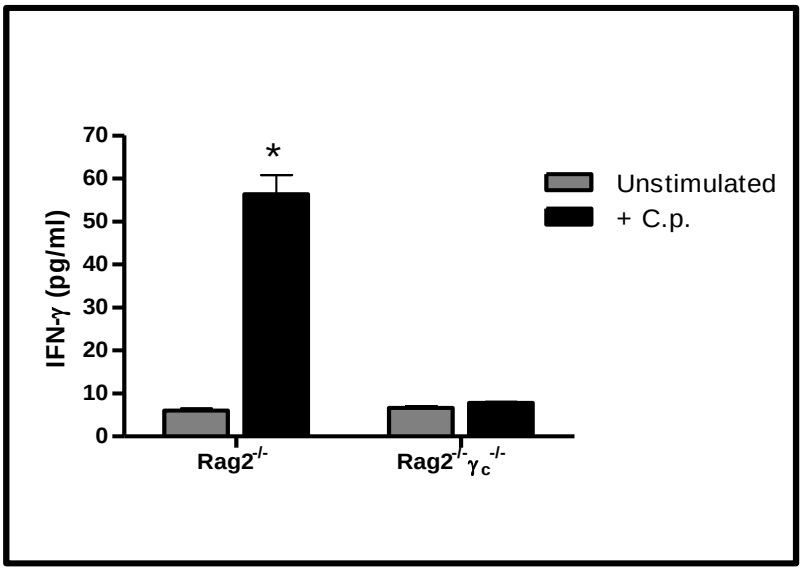

Figure 49.ELISA for IFN-γ production from cultured splenocytes from Rag 2-/- mice and Rag2-/-γc-/- after stimulation with *C. parvum* antigen.A significant amount of IFN-γ was found in supernatants from cells of Rag$2^{-/-}$ mice but not cells of Rag$2^{-/-}\gamma_c^{-/}$ mice (*p* <0.006)

4.3.3.4 *C. parvum* infection of neonatal Rag2$^{-/-}$ and Rag2γc$^{-/-}$ mice

In infection of adult Rag2$^{-/-}$ and Rag2γc$^{-/-}$ mice, apart from during the early acute phase it took a long period for the infection patterns to become significantly patent and to show consistent differences between the strains. As wild type neonatal animals are highly susceptible to infection with *C. parvum* (Harp *et al.,* 1990) it was of interest to examine and compare infections in neonatal Rag2$^{-/-}$ and Rag2$^{-/-}$ γc$^{-/-}$ mice.

4.3.3.4.1 Infection of 7 day old neonatal mice

7 day old Rag2$^{-/-}$and Rag2$^{-/-}$γc$^{-/-}$mice were infected with 5×10^4*C. parvum* oocysts and the level of infection was followed daily by counting parasites in stool smears. At day 4 postinfection both mouse strains had developed a patent infection. There was a significantly higher level of parasite shedding in the Rag2$^{-/-}$γc$^{-/-}$ mice on subsequent days up to day 9 (p<0.05). Interestingly, all mice from both groups survived the infection and, importantly, both mouse groups were able to control the infection strongly by day 11 and only a few parasites could be found by day 13 in stool samples obtained from Rag2$^{-/-}$γc$^{-/-}$ mice. Fig 50 summarises these results. As Rag2$^{-/-}$γc$^{-/-}$ mice took longer to recover, this result demonstrated that, as with adult mice, NK cells are important for recovery of neonatal T cell-deficient mice.

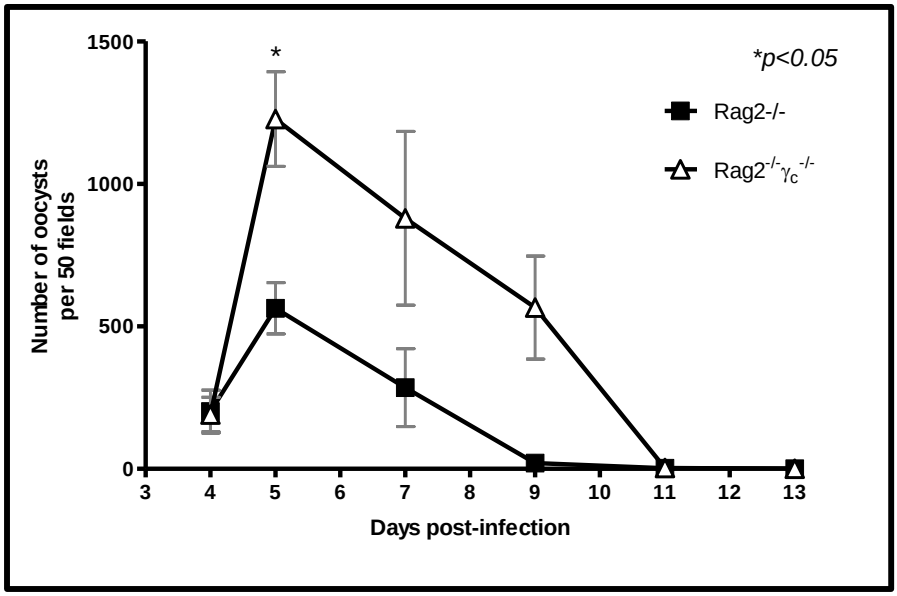

Figure 50.*C. parvum* infection in neonatal Rag2-/- and Rag2-/- γc-/- mice.

At days 7 of age Rag2$^{-/-}$ and Rag2$^{-/-}$γc$^{-/-}$ animals were infected by oral gavage with 5X10^{4} *C. parvum* and ocysts were counted in faecal samples on days shown.

4.3.3.4.2 Infection of 4 day old neonatal mice

It may seem surprising that neonatal Rag2$^{-/-}$γc$^{-/-}$ mice could survive infection, although they subsequently have the course of infection shown for adults (V.McDonald, unpublished data). As the mice were infected at 7 days of age it was considered possible that infecting mice at an earlier age, when the immune system would have been more immature might have produced more severe outcomes and show greater differences between Rag2$^{-/-}$ mice and Rag2$^{-/-}$γc$^{-/-}$ mice. Hence, patterns of infection were studied in mice infected at 4 days of age.

As shown in Fig 51, and similar to the previous experiment with older mice, for several days there was a higher level of oocyst production in Rag2$^{-/-}$γc$^{-/-}$mice when compared to the Rag2$^{-/-}$ mice, confirming a protective role for NK cells in immunity to this parasite

(p<0.04). Also, as before, both groups of mice were able to control the infection after an initial rapid rise in parasite reproduction. Cells other than NK cells, therefore, can contribute substantially to innate immunity when there is a lack of NK cells.

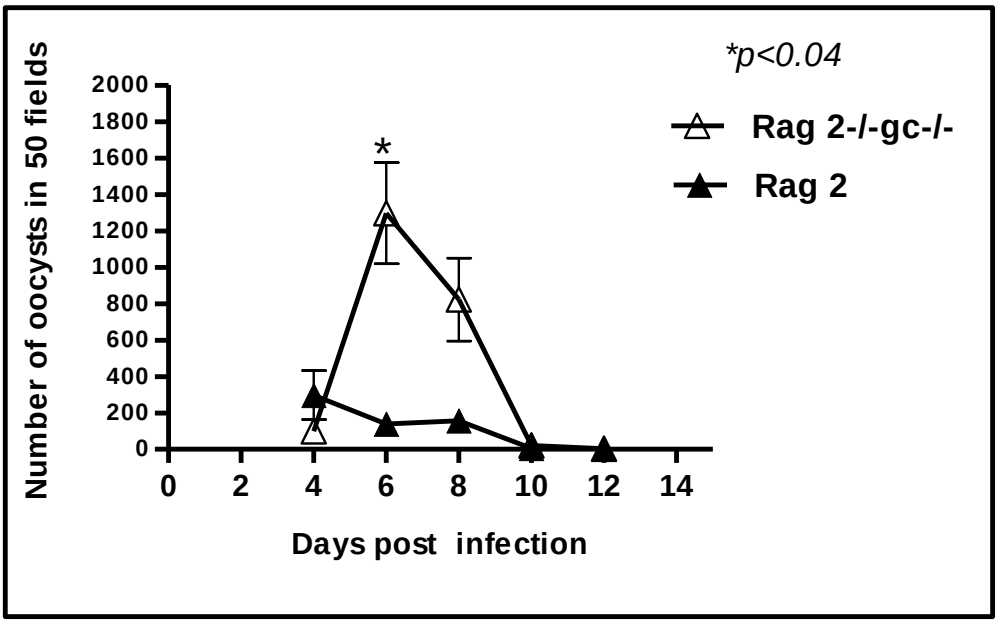

Figure 51.*C. parvum* infection in Rag2-/- and Rag2-/- γc-/-mice infected at 4 days of age.4 days old baby mice were infected with *C. parvum* oocysts and infection was measured by counting oocysts in stool smears on days shown.

4.3.3.5 The role of IFN-γ in recovery of neonatal mice from infection in the absence of NK cells.

4.3.3.5.1 IFN-γ expression in the intestine of neonatal Rag2$^{-/-}$ and Rag2$^{-/-}$ γc$^{-/-}$mice following *C. parvum* infection

The previous results indicated that in the absence of NK cells in Rag2$^{-/-}$ γc$^{-/-}$mice, there is an NK cell-independent mechanism to control the infection. Since IFN-γ is a key cytokine in controlling *C. parvum* infection in wild type mice and SCID mice, it was of great importance to measure this cytokine in the intestine of infected Rag2$^{-/-}$ and Rag2$^{-/-}$ γc$^{-/-}$mice. Neonatal Rag2$^{-/-}$ and Rag2$^{-/-}$ γc$^{-/-}$mice were sacrificed 7 days postinfection, during the recovery period, and ileal tissue samples were collected and used to measure the expression of IFN-γ by Q-PCR. Samples from uninfected animals of the same age were used as the calibrator.

There was only weak expression of IFN-γ mRNA in the uninfected mice (Fig 52) and, surprisingly, there was a significant level of mRNA expression in infected tissues from both mouse strains ($p \leq 0.01$*).* Indeed, the expression of IFN-γ in the Rag2$^{-/-}$ γc$^{-/-}$mice was stronger than in Rag2$^{-/-}$ mice, although this was not quite statistically significant (p=0.051). This result suggests that in the innate immune response against *C. parvum* infection there can be a major cellular source of IFN-γ other than NK cells.

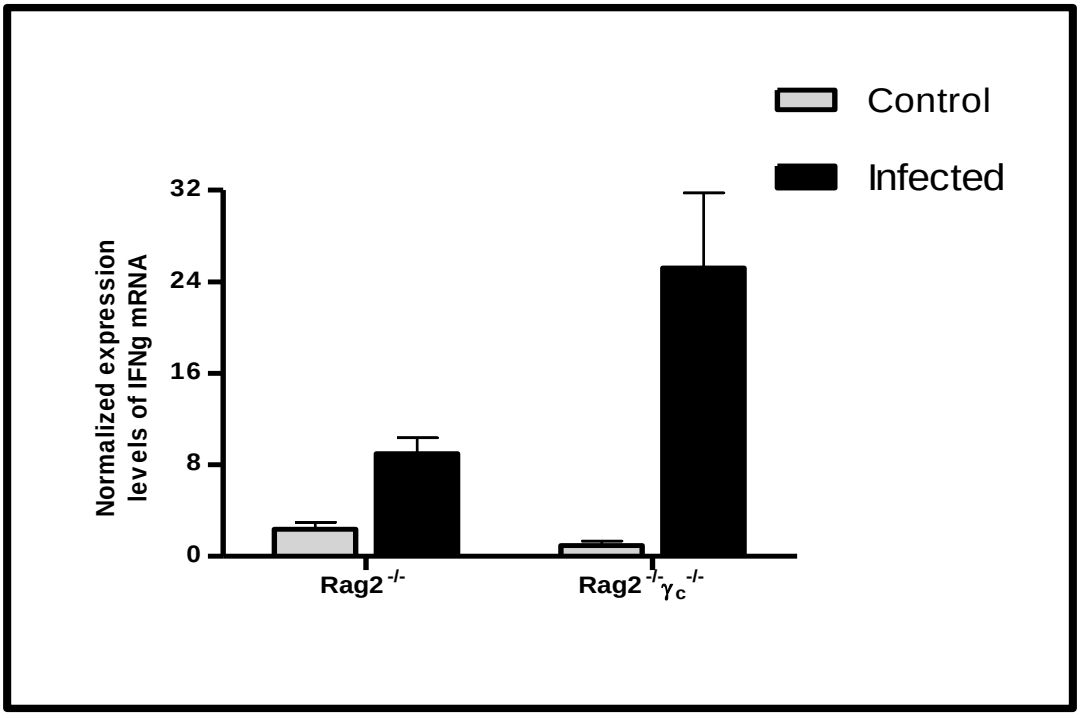

Figure 52.Intestinal IFN-γ expression in neonatal Rag2-/- and Rag2-/- γc-/- mice during acute *C. parvum* infection.Animals were infected by oral gavage at day 7 of age and intestinal tissue samples from the small intestine were collected 7 days post-infection. The amount of intestinal IFN-γ mRNA was quantified by real-time quantitative PCR. Infected tissues from both mouse strains expressed significant levels ($p \leq 0.01$) of IFN-γ compared to low levels detected in uninfected mice. Increased, but however insignificant level of IFN-γ was measured in Rag2-/- γc-/- ($p=0.051$).

4.3.3.5.2 The effect of IFN-γ neutralisation on infection of Rag 2$^{-/-}$ and Rag2$^{-/-}$γc$^{-/-}$ mice

In view of the previous result further experiments were carried out to determine if IFN-γ is essential for the control of infection, particularly in Rag 2$^{-/-}$γc$^{-/-}$ mice that lack NK cells. A study was performed comparing the effect of anti-IFN-γ neutralising antibody (H22 IgG mAb from hamster) on *C. parvum* infection in Rag 2$^{-/-}$ and Rag2$^{-/-}$ γc$^{-/-}$ mice.

Seven-days old mice from each strain were injected ip with either 100µg of anti IFN-γ antibody or PBS as a control since a non-reactive hamster IgG was previously shown to have no effect on parasite development (McDonald *et al.,* 1992; McDonald and Bancroft, 1994). Immediately afterwards all mice were infected with *C. parvum* and oocyst production was measured.

Figure 53 illustrates that, confirming previous observations, oocyst shedding in the control Rag2$^{-/-}$ γc$^{-/-}$ mice was greater than in Rag 2$^{-/-}$ mice controls, this time between days 4 and 8 postinfection. Treatment with anti-IFN-γ antibody resulted in an exacerbation of infection in both knockout mouse strains when compared to their controls (p<0.05). These observations indicate that IFN-γ played an important role in the ability of both Rag2$^{-/-}$ and Rag2$^{-/-}$γc$^{-/-}$ mice to control *C. parvum* infection. At the apparent peak of infection (day 6), the parasite count from antibody-treated Rag2$^{-/-}$γc$^{-/-}$ mice was significantly higher than from antibody-treated Rag2$^{-/-}$ mice (p<0.03). The infection level seen in the Rag2$^{-/-}$ γc$^{-/-}$ antibody-treated mice was the highest detected in all the *in vivo* experiments of these studies.

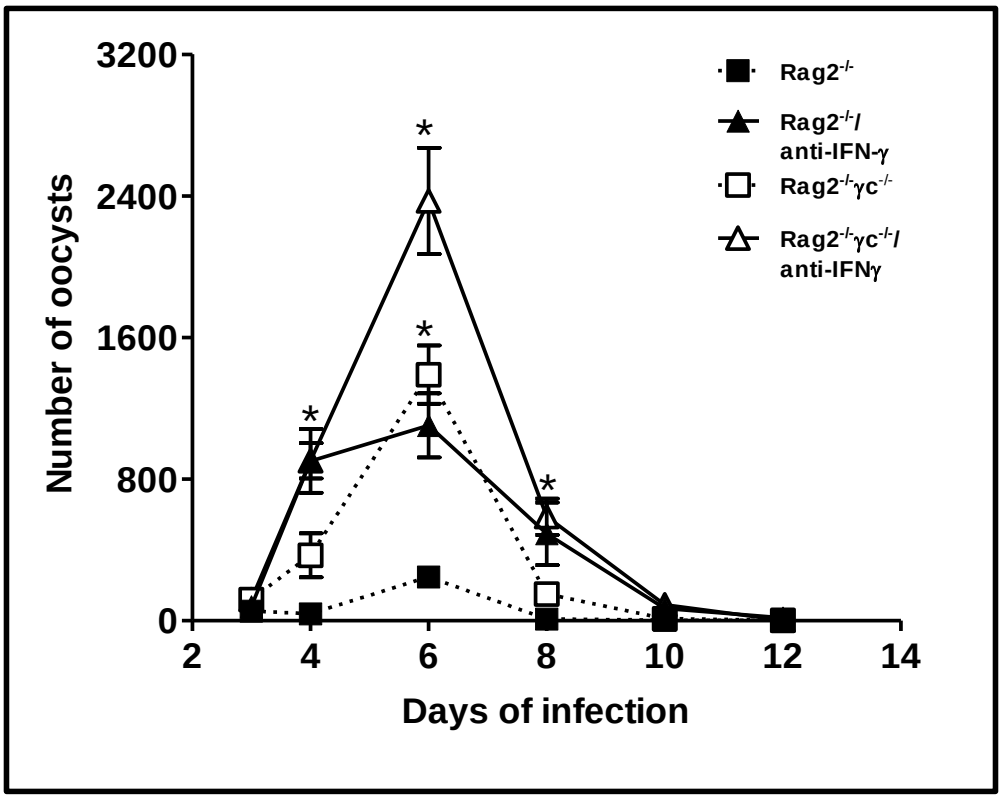

Figure 53.Effect of IFN-γ neutralisation on the course of *C. parvum* infection in neonatal Rag2-/- and Rag2-/- γc-/- mice. Seven day old mice were treated (ip) with antibody to IFN-γ or PBS as control prior to infection. The level of infection was followed by measuring oocyst shedding in the stool. Infection in both knockout mouse strains was intensified in the presence of anti-IFN-γ antibodies compared with controls (p<0.05). The parasite count at the peak of infection was significantly higher in antibody-treated Rag2$^{-/-}$γc$^{-/-}$ mice than in antibody-treated Rag2$^{-/-}$ mice (p<0.03).

4.4 DISCUSSION

NK cells are an important lymphocytic component of innate immunity. They are classically involved in the early defence against many viral infections and are also recognised for their anti-tumour role. They exhibit strong cytototxic activity and produce high levels of pro-inflammatory cytokines. Their role in immunity to different major protozoal diseases including toxoplasmoasis, trypanosomiasis, leishmaniasis and malaria is well recognised, particularly through the production of IFN-γ. Although NK cells can be activated in the presence of cryptosporidial antigen *in vitro* (although this had only been shown for *C. muris;* McDonald *et al.,* 2000) and despite suggestions from *in vivo* studies that these cells may be involved in innate immunity the exact protective role of these cells *in vivo* has not been clearly described.

With regard to NK cell cytotoxicity, human peripheral blood NK cells treated with IL-15 were shown to be cytotoxic against *C. parvum*-infected human epithelial cell lines and expression of this cytokine was detected in intestinal tissue samples of infected patients (Dann *et al.,* 2005). This is the only study to demonstrate NK cell cytotoxicity against cells infected by *Cryptosporidium*. Mice with the *beige* mutation that have NK cells deficient in cytotoxicity (Bannai, M *et al.,* 2000) developed heavier *C. parvum* infection than mice wild type mice, suggesting a protective role for cytotoxic NK cells (Enriquez and Sterling, 1991). However, the *beige* mutation has a similar effect on $CD8^+$ T cells although these cells have no major role in immunity (McDonald *et al.*, 2000).

The work described in this chapter can be divided into three sections based on the focus of the studies and involves NK functions both *in vitro* and *in vivo*.

The first section investigated IFN-γ production by cultured splenocytes stimulated by live *C. parvum* oocysts and the role of NK cells in expression of this cytokine. The

spleen cells came from uninfected SCID mice that lack T and B cells but have normal NK cells. In a cell culture assay employing 96-well plates, IFN-γ was produced but a minimum of $4X10^5$ splenocytes were required to produce a significant amount in the presence of $2X10^5$ oocysts. This result agrees with previous findings with *C. muris* in which it was shown that live sporozoites stimulated SCID mouse splenocytes to produce IFN-γ (McDonald *et al*., 2000). A previous study showed that *C. parvum* oocyst antigen induced IFN-γ production by splenocytes from SCID mice but the cells expressing IFN-γ were not characterised (Chen *et al.,* 1993). The cell numbers required to obtain substantial production of IFN-γ in the presence of *C. muris* was similar to that in the current study. Using $4X10^5$ splenocytes as the standard number in subsequent experiments the effect of varying oocyst numbers was examined. The highest level of cytokine production was obtained with $2x10^5$ oocysts and, interestingly, lower levels of IFN-γ were detected in supernatants from cells which were stimulated with higher number of oocysts. This observation might be explained by a higher degree of antigen stimulation inducing greater amounts of inhibitory factors including IL-10 or TGF-β that are known to reduce NK cell activity in infections (Hunter *et al.,* 1995; Lieberman and Hunter., 2002). Another possibility is that strong antigen stimulation induced NK cell apoptosis as has been shown with filarial antigen (Babu *et al.,* 2007). However, neither expression of inhibitory cytokines nor NK cell apoptosis was examined in this study.

Under the culture conditions employed at 37^oC most oocysts would release sporozoites within 1-2h but a question that had to be examined was the possibility that the oocyst shell was the prime stimulus for IFN-γ production. Live oocysts and an equivalent number of purified sporozoites (X4 as there are 4 sporozoites/oocyst) stimulated a similar level of IFN-γ production by splenocytes indicating that the sporozoites were the major stimulus for IFN-γ rather than the oocyst shell. Killing of sporozoites by freeze-thawing did not affect induction of IFN-γ, showing that viability does not affect

stimulation of cytokine production. These findings reflect observations with *Neosopora caninum* that heat-killed and live tachyzoites induced similar amounts of IFN-γ (Boysen *et al.,* 2006). A study with *Leishmania,* however, demonstrated that NK cells were only capable of producing IFN-γ after stimulation with live *Leishmania* promastigotes (Nylen *et al.,* 2003). One potential limitation regarding the splenocyte stimulation in the current work was possible. The experiments did not use a dead oocyst control. It is possible that the oocyst wall may have antigenic determinants that can stimulate splenocytes.

Activation of NK cell functions in response to infections is under the influence of different factors including cytokines and chemokines as well as direct cellular interaction with other cells, particularly DCs (Zucchini *et al.,* 2009; Cooper *et al.,* 2004). Different techniques have been employed to characterise *in vitro* the NK cell involvement in immunity to various microbial pathogens. Purification and depletion of the cells are valuable approaches and commonly used methods are the positive selection with magnetic microbeads conjugated with antibodies to NK surface markers or the use of antibodies to deplete NK cells such as the anti-asialo GM1 or anti-NK 1.1 antibodies (Souza-Fonesca-Guimaraes *et al.,* 2012).

In this study, NK cells were purified by positive selection with anti-CD49b microbeads and these cells were shown to be the likely major source of IFN-γ in response to *C. parvum*. On the other hand, negatively sorted cells depleted of NK cells failed to produce this cytokine in response to antigenic stimulation. These findings agree with previous results with *C. muris*; treatment of SCID mouse splenocytes with anti-asialo GM1 plus complement decreased production of IFN-γ in a dose-dependent manner (McDonald *et al.,* 2000). Positively selected NK cells were also shown to produce IFN-γ in response to *N. caninum* (Boysen *et al.,* 2006).

Positive selection of NK cells achieved a population that was only 80% $CD49b^+$ cells as demonstrated by FACS analysis, while nearly 100% of the negatively sorted population

were CD49b^{-}. Thus, there was the possibility that contaminating cells in the enriched NK cell population contributed to production of IFN-γ. In a previous study with *Leishmania*, complete purification of NK cells from human PBMC was achieved using magnetic negative selection and the cultured cells were directly stimulated to produce IFN-γ by parasite antigen. However, the exact mechanism of direct NK cell activation was not established in that study (Nylen *et al.,* 2003).

Cytokines such as IL-12, IL-15, IL-2, IL-1, IL-23, TNF-α and type I IFNs may stimulate NK cell activation in different infection models. (Lieberman and Hunter, 2002; Zucchini *et al.,* 2008). A study was made of the effect of neutralising antibodies to different cytokines on IFN-γ production by splenocytes. Treatment of cultured splenocytes with anti-IL-12 antibody decreased IFN-γ production induced by *C. parvum* oocysts in a dose-dependent manner. Stimulation of splenocytes with *C. parvum* oocysts also induced IL-12 production as measured directly by ELISA. These findings are in agreement with the report that IL-12 is important for intestinal IFN-γ expression in neonatal SCID mice infected by *C. parvum* (Urban *et al.*, 1996). IL-12 is a key factor of NK cell stimulation in response to other intracellular parasites, including *Toxoplasma gondii* (Hunter *et al.*, 1994). Many studies with *Leishmania* spp. also support the importance of IL-12 in NK cell activation (Schleicher *et al*., 2007), but a report by Nylen *et al.* demonstrated that IFN-γ production by cultured purified NK cells was independent on IL-12 (Nylen *et al.,* 2003).

Treatment with antibodies to TNF-α, IL-15 and IL-1, all examined in the same experiment, decreased IFN-γ production in a dose dependent matter. The most effective antibody was anti-TNF-α. A similar inhibitory effect with anti-TNF-α on IFN-γ release by NK cells was also detected in the *in vitro* study with *C. muris* (McDonald *et al.,* 2000). However, a study with neonatal TNF-α$^{-/-}$ mice in this laboratory suggested this cytokine was not necessary for the control of *C. parvum* infection (Lean *et al.,*

2006). Hence caution is required in extrapolating from *in vitro* data to the *in vivo* situation. In infection with *Listeria monocytogenes* TNF-α was found to act synergistically with IL-12 in its action to stimulate IFN-γ production by NK cells and was important for innate immunity *in vivo* (Tripp *et al.,* 1993).

Only moderate inhibition was obtained with anti IL-15, which can be important for NK cell activation during other infections (Chin *et al.,* 2009). A similar effect was obtained with anti-IL-1 that acts with other cytokines, including IL-12, to enhance NK cell activity (Hunter *et al.,* 1995). The role of IL-15 and IL-1 in the protective immune response to *C. parvum in vivo* is not known. Possible synergistic activity of these cytokines was not examined in the current study. It should be noted that the effectiveness of the neutralising antibodies could be a factor in the differences observed in these studies and the concentrations employed were based on preliminary studies with each antibody.

NK cells may be directly activated by type I IFNs produced by plasmacytoid DCs (a major source of these cytokines) during infection with viruses such as vaccinia (Martinez *et al.,* 2008) or can be indirectly activated by type I IFNs inducing DC and/or macrophage production of IL-15 that enhances NK cell priming after *in vivo* stimulation with LPS for *E. coli* or lymphocytic choriomeningitis virus (LCMV) (Lucas *et al.,* 2007). In the present study, using a bioassay to measure antiviral activity, significant levels of type I IFNs were detected in supernatants from NK cell-depleted splenocytes after stimulation with *C. parvum* indicating that in response to parasite stimulation ancillary cells are able to produce type I IFNs.These results strongly agree with the earlier findings from *in vitro* studies that bone marrow-derived DCs stimulated with oocysts showed significant expression of IFN-α IFN-β mRNA (Chapter 3). Type I IFN was shown to regulate IFN-γ expression by NK cells since reduced levels of IFN-γ were

obtained in cultured SCID mouse splenocytes treated with anti-type I IFN serum. Hence type I IFNs may directly activate NK cells and/or cause an increase in IL-12 expression (as in the *in vitro* experiments anti-type I IFN antibodies inhibited IL-12 production by splenocytes cultured with antigen).

The second section of this chapter investigated NK cell activity during the late phase of chronic infection of SCID mice. An early study by McDonald *et al.* indicated that chronic infection in adult SCID mice increased intensity only gradually over a period of weeks until ultimately mice showed morbidity and died after a few months of infection (McDonald *et al.,* 1992). Similar patterns of infection were also observed in SCID mice with the *beige* mutation and with adult nude mice (Mead *et al.,* 1991; Ungar *et al.*, 1990). An investigation was made of the activity of splenic NK cells in chronically infected mice showing signs of illness and in most cases a high number of oocysts (>70 oocysts/50 fields) were being shed in faeces.

While large amounts of IFN-γ were produced by splenocytes of uninfected mice, no IFN-γ was obtained from cells of age-matched severely infected mice. Interestingly, splenocytes from apparently healthy mildly infected mice (<10 oocysts/ 50 fields) produced more IFN-γ than cells from uninfected mice. This may have occurred as a result of modest systemic inflammation increasing numbers and/or activation status of NK cells as well as macrophages and DCs at this stage of infection. No study of possible mechanisms was made, however.

Cryptosporidium infection in immunocompromised patients can affect the entire intestine and spread to extraintestinal sites such as the pancreas, hepatic bile ducts and gall bladder. (Rosario de Souza *et al.,* 2004; Davies and Chalmers, 2009). Hepatobiliary infection and disease has been described in immunocompromised mice including SCID mice and SCID mice with the *beige* mutation causing death in those

animals often as a result of hepatic dysfunction (Mead *et al.,* 1991). In the current study, a similar presentation of infection was observed in many of the chronically infected mice. Some mice with low-modest oocyst shedding in the stool, however, were clearly unwell and jaundiced and the histopathological examination of the liver tissue confirmed parasites in the bile duct epithelium. As with ill mice that shed many oocysts in faeces, splenocytes of the mice with hepatobiliary disease but low level of oocyst shedding also failed to produce IFN-γ in response to *C. parvum* stimulation.

It was important to investigate further the possible cause/s for the defect of IFN-γ production observed in splenocytes of mice in the late stage of infection. As NK cells were identified as the main source of IFN-γ in stimulated splenocytes it was therefore critical to examine the NK cell population in the spleen and the intestine of the severely infected mice. Interestingly, no NK cells were identified in splenocytes of severely ill mice when examined by flow cytometry. This presumably explains the absence of IFN-γ production, therefore, in the spleen of these mice.

The possible reason/s for the absence of $CD49^+$ cells in the present study could be: 1) The increasing level of infection caused systemic exhaustion of the NK cell population as a result of prolonged activation of macrophages producing excessive amounts of IL-12 that causes global NK cell depletion as seen in macrophage activation syndrome (Ravelli *et al.,* 2002). Kinetic analysis of these cytokines in relation to IFN-γ and numbers of splenic NK cells could provide evidence of what is happening. 2) NK cells and/or other cells such as DCs cells migrate from the spleens to the intestine as the infection intensifies. 3) As may occur during some viral infections (Zou *et al.,* 2005, Voigt *et al.,* 2007), there is down-regulation of NK cell receptors (perhaps including CD49b) induced by persistent infection or parasite mediators providing a strategy for

the parasite to avoid host immune responses. 4) Induced NK cell apoptosis may occur late during infection.

Further to these findings it was essential to examine the NK cell population in the intestine of infected mice and their ability to produce IFN-γ. A technique previously described for isolation of intraepithelial NK cells from mice infected with *Trichinella spiralis* was employed (McDermott, *et al.,* 2005), but all attempts to isolate NK cells from the intestines of healthy or ill mice failed. Therefore, it was not possible in this study to examine the NK cells or their function in the intestine of the infected mice. Results of other studies in this lab to measure IFN-γ mRNA expression in the intestine of the severely infected $Rag2^{-/-}$ mice were inconclusive: the cytokine was detected at relatively high levels in some mice but was not detected in others (D.S. Korbel and V. McDonald, unpublished data).

NK cell populations in the spleens of the moderately infected mice was not investigated in the existing study.

The third section of this Chapter returned to examining the role of NK cells in innate immunity *in vivo*. Previous studies with T cell-deficient mice have only implied that NK cells are important in immunity against *C. parvum* (McDonald *et al.*, 2000). Significantly, NK cell depletion using anti-asialo GM1 antibodies in BALB/c SCID mice failed to show any effect on the course of infection of *C. parvum* (McDonald and Bancroft, 1994; Ungar *et al.,* 1991). Although this technique is well recognised for NK cell depletion, it has been suggested that these antibodies might not have reached the gut in sufficient quantity to be effective, despite the successful depletion seen with other studies involving intestinal NK cells (Yoshihara *et al.,* 2006). It is possible that an NK cell subpopulation that does not express asialo GM1 is involved in immunity. Also, NK cell depletion using this technique should be interpreted cautiously as these antibodies

were shown to affect other innate immune cells including basophils (Nishikado *et al.,* 2011).

Comparative studies with Rag2$^{-/-}$ and Rag2$^{-/-}$γc$^{-/-}$ mice were previously shown to be valuable for studying NK cells in immunity to other infections. In *Shigella* infection adult Rag2$^{-/-}$γc$^{-/-}$ mice died after a few days while Rag2$^{-/-}$ mice survived (Le-Barillec*et al.,* 2005). Furthermore, a study with*P. yoelii* indicated that Rag2$^{-/-}$γc$^{-/-}$ micewere significantly more sensitive to infection with *P. yoelii* sporozoites than Rag2$^{-/-}$ mice (Roland *et al.,* 2006). In the current study, therefore, the pattern of infection and immune responses were studied in adult as well as neonatal Rag2$^{-/-}$ and Rag2$^{-/-}$γc$^{-/-}$ mice.

Infection of weaned Rag2$^{-/-}$ and Rag2$^{-/-}$γc$^{-/-}$ mice followed a progressive chronic pattern similar to that described with adult SCID mice. The fulminant stage of infection developed sooner in Rag2$^{-/-}$γc$^{-/-}$ mice than in Rag2$^{-/-}$ mice, however, and when the former mice showed signs of morbidity they developed histopathological changes in the intestine. All Rag2$^{-/-}$γc$^{-/-}$ mice died whereas at the end of the monitoring period all Rag2$^{-/-}$ mice were still alive and appeared healthy. These results indicate that NK cells are important in the innate immune response against *C. parvum.* However, the ability of Rag2$^{-/-}$γc$^{-/-}$ mice to resist the infection for numerous weeks suggests that there are NK cell-independent mechanisms in innate responses to this parasite. Supporting these results, Flow cytometry analysis of the spleen cells of uninfected Rag2$^{-/-}$ mice contained a substantial number of CD49b^{+} NK1.1^{+} cells, indicative the presence of NK cells whereas no NK cells were detected in the spleens of Rag2$^{-/-}$γc$^{-/-}$ mice (Korbel and McDonald, unpublished data).

Neonatal animals including mice, sheep, cattle and deer are highly susceptible to *C. parvum* infection while immunocompetent adult animals (but not humans for unknown reasons) have very low levels of infection (Fayerand Ungal*,* 1986). It was of interest,

therefore, to examine the course of *C. parvum* infection in newborn Rag2$^{-/-}$ mice and Rag2$^{-/-}$γc$^{-/-}$ mice to determine whether wide differences between strains in susceptibility to infection might be evident. When mice were infected at the age of 7 days, both mouse strains developed heavy levels of infection, but Rag2$^{-/-}$γc$^{-/-}$ mice were found to produce significantly higher numbers of oocysts for several days. Importantly, despite the high levels of infection observed, both groups were able to overcome this early phase of infection and established strong control and there was a low rate of mortality in both strains. When mice were infected at 4 days of age, similar results were obtained, including a low rate of mortality. The ability of these mice to recover may partially be due to the onset of age-related "natural" resistance to this infection seen in different mammalian species, the basis of which is unclear (Sherwood *et al.,* 1982). However, as in SCID mice infected as neonates, relapse of infection and death occurred several weeks later and was observed sooner with Rag2$^{-/-}$γc$^{-/-}$ mice (V. McDonald, unpublished data).

The increased susceptibility to infection observed in the young Rag2$^{-/-}$γc$^{-/-}$ mice compared to the Rag2$^{-/-}$ mice suggests that NK cells play an important protective role against *C. parvum* in neonates as well as adults. In agreement with this, recent work in this laboratory also showed that treatment of neonatal wild type C57BL/6 mice with anti-NK1.1 antibodies increased their susceptibility to infection as confirmed by higher levels of infection in comparison to the control mice (Korbel *et al*., 2011). Moreover, the same study observed that during the early acute phase of infection over about 12 days, Rag2$^{-/-}$ mice were not more susceptible to infection in comparison to the wild type mice. This study not only supported a role for NK cells in the protective immune response of neonatal wild type mice, but also caste doubt about the role of T cells in neonatal immunity against *C. parvum* (Korbel *et al.,* 2011).

As it is well established that IFN-γ is a key cytokine in innate immunity to this parasite, the role of this cytokine in control of infection in Rag2$^{-/-}$ and Rag2$^{-/-}$ γc$^{-/-}$mice was investigated. Interestingly, it was shown that infection of both neonatal Rag2$^{-/-}$ and Rag2$^{-/-}$γc$^{-/-}$ mice stimulated IFN-γ expression in the intestine. Also treatment of both Rag2$^{-/-}$ and Rag2$^{-/-}$γc$^{-/-}$ mice with anti-IFN-γ neutralizing antibodies exacerbated infection. These findings indicated that this cytokine played an important part in the ability of both mouse strains to control the infection. Similar findings were observed in a recent study in this laboratory of *C. parvum* infection of adult Rag2$^{-/-}$γc$^{-/-}$ mice. Repeated administration of anti-IFN-γ neutralizing antibodies to those animals abolished their resistance during early infection with this parasite (Choudhry *et al.,* 2012)

This indicated that in the *in vivo* infectionthere can be a major cellular source of IFN-γ in the absence of NK cells. This contradicts the *in vitro* findings described in this Chapter that showed splenocytes from SCID mice depleted of NK cells or from the Rag2$^{-/-}$γc$^{-/-}$ mice were incapable of IFN-γ production in response to stimulation with parasite antigen. However, it is possible that in the intestinal physiological environment interaction between different immune cells and factors from infected epithelial cells may induce IFN-γ production. A recent report by others from this laboratory showed that the infected (but not uninfected) mouse intestinal epithelial cell line CMT-93 treated with IFN-γ produced large amounts of IL-18 (Choudhry et al., 2012).

That same study also showed that *in vitro* the presence of both IL-18 and IL-12 induced large amounts of IFN-γ production by Rag2$^{-/-}$γc$^{-/-}$ splenocytes or purified peritoneal macrophages, but neither cytokine alone could do so. It was also shown that *C. parvum* infection in adult Rag2$^{-/-}$γc$^{-/-}$ mice increased rapidly following macrophage depletion and this correlated with no increased IFN-γ expression in the intestine. Also, IFN-γ production in the Rag2$^{-/-}$γc$^{-/-}$ mice was dependent on IL-18 as anti-IL-18 neutralising antibody treatment exacerbated infection and inhibited the IFN-γ response (Choudhry

et al., 2012). Whether this IL-18-dependent immunity involving macrophages is important when NK cells are present is not known. In support of these findings, Takeuchi *et al.* demonstrated that the transference of peritoneal macrophages from *C. parvum*-infected mice to X-irradiated SCID beige mice depleted of macrophages allowed the recipients to survive *C. parvum* infection whereas macrophages from uninfected animals were not protective. Additional *in vitro* work suggested that IFN-γ released by neutrophils may have played a role in macrophage activation (Takeuchi *et al.,* 2008). However, in our laboratory neonatal mice with antibody-mediated neutropenia did not have increased susceptibility to infection (D.S. Korbel and V. McDonald, unpublished data).

Previous studies with bacterial pathogens such as *Salmonella* have indicated that IFN-γ production by macrophages and neutrophils was important in the early innate immune response to infection (Kirbry *et al.,* 2002). In pulmonary *Chlamydia* infection of mice macrophages were also capable of expressing IFN-γ that was required for the innate immune defence of immunocompromised mice, although in contrast to the current study, there was no difference in the susceptibility to infection of $Rag2^{-/-}$ and $Rag2^{-/-}\gamma c^{-/-}$ mice (Rothfuchs *et al.,* 2004). Dendritic cells could also play a role in this respect as it has been shown previously that these cells were a potent source of IFN-γ when activated by IL-12 and IL-18 (Lugo-Villarino, *et al.,* 2003).

Even with a high level of IFN-γ expressed in the intestine of $Rag2^{-/-}\gamma c^{-/-}$ mice, these animals eventually developed fulminant infection and died. The reasons for this are unclear but it could possibly be related to increased production of IL-10 as infection progresses (Choudhry *et al.,* 2012). Chronic inflammation might eventually alter the intestinal bacterial composition that may influence the nature of the inflammatory response. It is also possible that during chronic infection the parasite develops increased virulence, but this has not been investigated.

In conclusion, it was shown that *C. parvum* induced IFN-γ production by NK cells *in vitro* and this was regulated by different pro-inflammatory cytokines including IL-12, IL-15, IL-1, TNF-α and type I IFNs. The results from mouse experiments also indicate that NK cells are important in the innate immune responses to *C. parvum* infection of immunocompromised mice. IFN-γ plays a central part in innate immunity in mice and NK cells are normally a major source of this cytokine, but in the absence of NK cells other cells, probably macrophages, can produce this cytokine as suggested by studies by other colleagues in this laboratory. During the late stage of chronic infection of immunocompromised mice a defect in the splenic NK cell population was described that if occurred in the intestine and liver could explain the death of the animals due to *C. parvum*.

CHAPTER FIVE

FINAL DISCUSSION AND FUTURE WORK

Evidence from studies of protective host immune responses to *Cryptosporidium* indicates a major role for cellular immune responses in elimination of infection (McDonald, 2000). In this regard, $CD4^+$T cells play a dominant role in completing resistance to *C*. *parvum*. In addition to $CD4^+$Tcells, T_H1 cytokines, particularly IFN-γ, play a key role in the control of infection (Ungar *et al.,* 1991; Aguirre *et al.,*1994). Murine *and in vitro* studies with *C. parvum* also strongly support a significant role for innate immune responses in the control of infection (McDonald *et al.,* 2013). Parasite invasion of enterocytes activates TLR/NF-KB signalling, resulting in the production of cytokines, chemokines, prostaglandin E2, antimicrobial peptides and nitric oxide, all of which may help in inhibiting parasite reproduction (Zhou *et al*., 2102; McDonald, 2011; McDonald *et al.,* 2013). Myeloid cells including macrophages, dendritic cells, and neutrophils also have involvement in the innate responses to this parasite (McDonald*,* 2007; McDonald *et al.,* 2013). In the current study the roles of NK cells, a major source of IFN-γ, and type I IFN in the innate immune response to *C. parvum* are explored.

Type I IFNs are a multigene family of cytokines with pleiotropic effects (Isaacs and Lindenmann, 1957). Their central role as inducers of antiviral host responses is well established (Takaoka and Yanai, 2006), and most nucleated cell types infected by virus are believed to be capable of expressing type I IFN (Koyama, 2008). They act alone and with other cytokines to clear infection (Koyama, 2008). These cytokines also have various effects on immune cells (for example, stimulation of antigen presentation and activation of T cells and NK cells) (Fitzgerald-Bocarsly and Feng, 2007). Apart from their strong antiviral activity, type I IFNs have been found to play roles in the immunity against some bacterial, protozoal and fungal infections, but not others (Bodgan *et al*, 2004).

The role of type I IFN in immunity to parasitic protozoa is less well described, although it is clear that they play an important role in reducing the severity of

infection due to some species, such as *Leishmaniamajor* (Mattner *et al*., 2004). The role of type I IFNs in immunity to the many enterocytic apicomplexan parasites, such as *Cryptosporidium* species, has not previously been investigated. This is the first study to investigate the role of type I IFN in immunity to *Cryptosporidium* and to show that type I IFNs inhibit *C. parvum* development both *in vitro* and *in vivo.*

Treatment of intestinal epithelial cell lines with IFN-α/β decreased parasite reproduction and this anti-parasitic effect was obtained with both human and murine lines and was therefore not specific to one host type.

Different antimicrobial killing mechanisms have been associated with type I and type II IFN activity against intracellular microbial pathogens. Inhibition of cell invasion is a known mechanism of type I and type II IFN antimicrobial activity (Niesel *et al.*, 1986). In the current study, IFN-α prevented host cell invasion by *C. parvum* as indicated by significant reduction of parasite numbers observed in IFN-α-treated cells 3 h postinfection. However, the exact mechanism of how type I IFN inhibited parasite invasion was not identified in this study. In a study with *Shigella flexneri* infection, IFN-α inhibited a Src-dependent signaling cascade triggered by *Shigella* that leads to the reorganization of the host cell cytoskeleton and bacterial entry into the cell (Dumenil *et al.,*1998). In *C. parvum* infection, Forney *et al,*had shown that inhibition of cytoskeletal rearrangement in host cell cells with a drug may prevent invasion of the host cell by the parasite (Forney *et al.,* 1999). However, in the present project the effect of type I IFNs on proteins known to be engaged in cytoskeletal organisation was not examined and it would be essential to undertake further studies in that area in future.

Other recognised antimicrobial mechanisms that were previously associated with type I or type II IFN such as deprivation of cellular Fe^{2+} or of L-tryptophan or NO production were also studied and were found not relevant for type I IFN activity

against *C. parvum.* Furthermore, unlike a previous finding with IFN-γ (Pollok *et al.,* 2000), IFN-α did not appear to affect the rate of maturation of intracellular parasites.

Employing different techniques it was shown that enterocytes as well as dendritic cells (major type I IFNs producing cells) are capable of expressing type I IFNs in response to *C. parvum*. Also the treatment of enterocytes with the TLR3 agonist poly (I:C) or infection with *C. parvum* stimulated the production of type I IFNs. Furthermore, infected CMT-93 cells also secreted products that further inhibited parasite development; those products might include IFN-α/β. The nature of the products was not identified in the current study and it is therefore important that in future the possible involvement of IFN-α/β be examined. It is known that type I IFN is produced via TLR(s) (Doyle *et al.,* 2002; Hemmi *et al.,* 2003) and as indicated by a few studies TLR4 in epithelial cells is activated by *C. parvum* (O'Hara *et al.,* 2011, Zhou *et al.,* 2012) it is possible therefore that type I IFN is I induced via TLR4 in response to *C. parvum.* Hence it is important to carry out further work to explore the role of TLRS for induction of type I IFN expression in enterocytes.

The results from the current study along with another recent observation (Bedi and Mead, 2012) suggest that DCs are capable of cytokine production (including type I IFNs and IL-12) in response to *C. parvum* stimulation. Further work on the role(s) of DCs in the immunity to this parasite is required.

Following the findings of type I IFN activity *in vitro* it was important to proceed by studying the *in vivo* role of type I IFNs in innate immunity to *C. parvum.* The IFNAR$^{-/-}$ knockout mice have been previously successfully used by different groups studying the role of type I IFNs in other infection models. However, for unknown reasons, in this laboratory the commercially available 129SV/Ev wild type and IFNAR1$^{-/-}$ neonatal mice were both found naturally resistant to infection with the parasite (V. McDonald and G. Foster, unpublished results). To overcome this problem, anti-IFN-α/β neutralising antibodies were employed in the present project to study the role of type I IFNs in immunity to *C. parvum*. A significant finding was that treatment with

anti-IFN-α/β enhanced the infection in both immuncompromised and immunocomeptent neonatal mice, indicating a crucial role for type IFNs in protection against *C. parvum in vivo.* Furthermore, it was shown that type I IFNs inhibited parasite development at an early stage of the infection and both IFN-α and IFN-β had increased expression in the intestine of infected animals within 24-48h of infection. Expression of these cytokines in the later stages of infection was not examined in the current study and it may be useful to address that in the future.

Different mechanism(s) of action are described for type I IFNs in other models of infection including the stimulation of IFN-γ production (Orellana *et al.,* 1991) which is an essential cytokine in immunity to *C. parvum*. However, it appeared that the protective effect observed with type I IFN was independent on IFN-γ production as treatment with anti-type I IFN did not prevent an increase in IFN-γ expression. Other possible mechanism(s) were not investigated. Moreover, any nucleated cell can produce type I IFN in response to infection, but the cell types producing type I IFNs in mice were not identified and it is therefore essential to conduct further studies in the future to investigate this point.

Although exogenous treatment with type I IFN has previously enhanced protection against other microbial infections, in the current study the treatment of SCID mice with exogenous IFN-α did not cause any alteration in the level of infection. Different technical reasons could explain this negative finding such as quantity of cytokine employed and number of times of administration and further studies will be required on this topic.

NK cells are key cellular components of innate immunity involved in resistance to intracellular microbial pathogens. Some early studies suggested there was involvement of NK cells in immunity to *C. parvum,* but the exact role of these cells has not been identified. Using different strains of immunocompromised mice, the role of NK cells in the acute infection of neonates and chronic infection of adults as well as their *in vitro* activity was studied in the present project.

It was confirmed by flow cytometry that splenocytes from healthy uninfected adult SCID mice contained normal numbers of NK cells and in response to stimulation with *C.parvum* antigen, SCID mouse splenocytes were capable of IFN-γ production. NK cells were identified as the major cellular source of this cytokine after the positive magnetic isolation of these cells. Similar findings were observed with studies with splenocytes from Rag2$^{-/-}$ mice that produced IFN-γ but cells from Rag2$^{-/-}$ γc$^{-/-}$ mice that lack NK cells did not. Supporting these findings, previous studies with the gastric parasite *C. muris* also showed that live sporozoites stimulated SCID mouse splenocytes to produce IFN-γ and this was inhibited by treatment with antibody plus complement to deplete of NK cells in a dose dependent matter (McDonald *et al*., 2000). In the current study, the optimal level of IFN-γ production was observed in cells stimulated with 2x10^5 oocysts, but lower amounts of cytokine were produced from cells stimulated with higher numbers of oocysts. This may suggest the presence of inhibitory mechanism(s) that switched off NK cell activity, but this was not investigated further. Further work should be done to determine if this is an important issue that may also affect cytokine production *in vivo*.

Cytokines such as IL-12, IL-15, IL-2, IL-1, IL-23, TNF-α and type I IFNs stimulate NK cell activity (Zucchini *et al.,* 2008). In the current study, *in vitro* IFN-γ production by NK cells was controlled by different cytokines including (IL-12, IL-15, IL-1, TNF-α and type I IFNs) as confirmed by reduced levels of IFN-γ from splenocytes treated with neutralizing antibodies to these cytokines. IL-1 and IL-15 appeared to be only modest inducers of IFN-γ production, however, under the culture conditions employed. Further studies are required to establish how important these cytokines might be for immunity *in vivo*. TNF-α appeared to be important for inducing IFN-γ in culture, but was shown to be nonessential for immunity in experiments with TNF-α$^{-/-}$ mice done previously in this laboratory, so caution is required in interpretation of the results of these *in vitro* studies of the regulation of IFN-γ production by NK cells.

Cryptosporidiosis is a chronic life threatening disease in immunocompromised patients and those patients are at higher risk of developing extraintestinal infection (Davis and Chalmers, 2009). A similar picture of infection was previously observed in *in vivo* studies of chronic cryptosporidiosis of immunocompromised mice. The infection increased gradually over weeks and the eventual severe infections were associated with clinical deterioration followed by death. Some animals developed hepatobilliary infection that led to hepatic impairment and eventually death (McDonald *et al.,* 1992; Ungar *et al,* 1990; Mead *et al.,* 1991).

In the present study the activity of NK cells in the chronically infected SCID mice showing signs of morbidity and often shedding high numbers of oocysts or with hepatic involvement was assessed. Results indicated that in severely ill animals, splenocytes were incapable of IFN-γ production and this was associated with absence of the $CD49b^+$ NK cell population in the spleen. It was planned to study the NK cell population from the intestine of severely ill mice, but attempts at isolating these cells were not successful in the current project. Further studies are required to explain the (apparent) lack of splenic NK cells observed in ill animals and a method is required to detect NK cell in the gut, perhaps by immunohistology. Attempts have been made to identify NK cells or IFN-γ-producing cells in the gut histologically in this laboratory but without success (V. McDonald, unpublished work).

One of the possible mechanisms proposed for NK cell depletion is a generalised exhaustion of NK cells caused by activation of macrophages and the release of large amounts of IL-12, a picture similar to what is seen in “macrophage activation syndrome”, that often affects young patients. Analysis of other cytokines, particularly IL-12 in relation to IFN-γ production and the number of NK cells could explain this. Other possible mechanisms for NK cell inactivation or depletion including downregulation of the CD49b marker, NK cell induced apoptosis and migration of NK cells to the site of infections, should all be considered as areas of work.

Splenocytes of animals with mild infection during the early weeks of infection produced higher levels of IFN-γ in comparison to uninfected mice. This could be due to a systemic inflammatory process with possible increases in numbers of NK cells and ancillary cells. The NK cell population in the spleens of the moderately infected mice was not investigated in the existing study. Therefore, it would be important to examine this in future and to determine if the inactivation/depletion of NK cells in the spleen is a gradual or a sudden event. Also, the functions of NK cells, macrophages and DCs should be compared at different stages of infection of SCID mice. Ideally, similar studies should be done with intestinal immune cells.

Better understanding of the loss of NK cell fuction associated with severe *C. parvum* infection could help explain some of the immunological causes of chronic diseases in humans and may offer the basis for designing therapeutic options for some microbial and autoimmune conditions.

Previous reports with immunocompromised T cell deficient mice have only indirectly suggested that NK cells are important in immunity to *C. parvum* (McDonald *et al.,* 2000). The main evidence was that IFN-γ was essential for the control of infection in SCID mice (McDonald and Bancroft, 1994). Studies of the effect of NK cell depletion on infection in SCID mice by administration of anti-asialoGM1 antibodies failed to show a protective role for these cells. Therefore, using a different approach, $Rag2^{-/-}$ mice that have functional NK cells and $Rag2^{-/-}\gamma c^{-/-}$ mice that lack these cells were employed in the current project. Adult $Rag2^{-/-}\gamma c^{-/-}$ mice, like $Rag2^{-/-}$ mice, showed resistance to infection for several weeks. However, fulminating infection, intestinal pathology as well as mortality occurred sooner in $Rag2^{-/-}\gamma c^{-/-}$ mice. The infection pattern was similar to the one observed previously with adult SCID mice. With neonatal animals, $Rag2^{-/-}\gamma c^{-/-}$ mice were, remarkably, able to control infection but developed higher levels of oocyst reproduction than $Rag2^{-/-}$ mice. A low rate of mortality observed in neonatal mice of both strains.

The increased susceptibility to infection observed in Rag2$^{-/-}$γc$^{-/-}$ mice compared to the Rag2$^{-/-}$ mice suggests that NK cells play an important protective role against *C. parvum* in neonates as well as adults. Supporting this, a recent study in this laboratory indicated that treatment of neonatal wild type C57BL/6 mice with anti-NK1.1 antibodies increased their susceptibility to infection (Korbel *et al.,* 2011).

Unlike the *in vitro* findings of this study that confirmed NK cells as a major source of IFN-γ in the immunocompromised animal, in the *in vivo* model significant levels of the cytokine were detected in the intestine of animals lacking NK cells as well as animals with NK cells, suggesting NK cell independent sources of this cytokine. In agreement with this finding, other researchers have recently described the ability of macrophages to produce IFN-γ in response to *C. parvum* infection in the absence of NK cells (Choudhry *et al.,* 2012; Takeuchi *et al.,* 2008).

Furthermore treatment of neonatal animals with anti-IFN-ã neutralizing antibodies exacerbated infection in both Rag2$^{-/-}$ and Rag2$^{-/-}$γc$^{-/-}$. Similarly, recently in this laboratory it was shown that repeated administration of anti-IFN-ã neutralizing antibodies to adult Rag2$^{-/-}$γc$^{-/-}$overcame their early resistance to infection with this parasite. The same study demonstrated IFN-γ-mediated immunity was dependent on IL-18 (Choudhry *et al.,* 2012). Together, these findings confirm the importance of this cytokine in the control of the infection in both mouse strains.

Despite the high level of IFN-ã expressed in the intestine of Rag2$^{-/-}$ãc$^{-/-}$ mice, these animals eventually developed severe infection and died. The reasons for this are unclear but it could possibly be related to increased production of IL-10 as infection progresses (Choudhry *et al.,* 2012). Therefore, further investigations in this area are suggested.

Regarding the cytolytic activity of NK cells, evidence suggests that NK cell cytotoxicity might have a role in immunity to *C. parvum* infection. One study has shown that human peripheral blood NK cells activated by IL-15 had cytolytic activity

against enterocyte cell lines infected with *C. parvum* and, in addition, infected epithelial cells expressed MICA, a ligand for the NK cell activating receptor NKG2D (Dann *et al,* 2005). Also, extraintestinal cryptosporidial infection was observed to be less common in SCID mice than in similar mice that also carried the *beige* mutation that causes different deficiencies, including in NK cell cytotoxicity (Mead *et al.,*1991). In the current study the cytolytic activity of NK cells was not examined and further work is required in this aspect.

In conclusion, results from this project provide new evidence for the importance of the innate immune response against *C. parvum.* It indicates that: 1) NK cells in immunocompromised mice are important to sustain the innate control of *C. parvum* infection. It was confirmed that IFN-γ plays a key part in maintaining innate immunity but a cell type(s) other than NK cells that produces the cytokine is also notably involved. It is therefore necessary to characterise the cell types expressing IFN-γ in T cell-deficient and alymphocytic mouse strains.During the late stage of chronic infection of immunocompromised mice a defect in the splenic NK cell population was described that if also present in the intestine could explain the death of the animals due to *C. parvum*.

2) In this, the first investigation of the role of type I IFNs in immunity to *Cryptosporidium* species, it was established that *C. parvum* infection induced rapid intestinal expression of IFN-α/ β and that these cytokines were involved in the protective innate immune response. Both enterocytes and dendritic cells were shown to produce these cytokines *in vitro*, and importantly, IFN-α/β directly inhibited parasite development in enterocytes and the principle mechanism of cytokine action appeared to be to prevent parasite invasion of enterocytes.

Different clinical applications have been previously described for type I IFNs particularly in the treatment of viruses (D'Souza and Foster, 2004b, D'Souza and Foster 2004a; Casrouge *et al.,* 2006). The findings in the present study could offer

the basis for future therapeutic approaches to control infection by different microbial agents including *Cryptosporidium*.

Findings from this project may contribute to an understanding that will possibly in the future lead to the management of the chronic cryptosporidiosis in immunocompromised patients. They will potentially have relevance to some other medical conditions, particularly autoimmune diseases.

Further human studies in the immune response to this parasite particularly in innate immunity are required including the role of TLR-mediated pathways in human intestinal epithelial cells. Also the role of NK cells, dendritic cells and macrophages in innate responses to *Cryptosporidium* could be examined. Investigation of the role of chemokines and cytokines in innate and adaptive responses in humans would also be valuable and could be facilitated by the use of newer technologies that require only small volumes to quantify several cytokines in the same sample. Findings from future studies may offer the basis for therapeutic options particularly for patients who suffer with immunodeficiency.

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APPENDIX

List of Publications

McDonald V, Korbel DS, Barakat FM, Choudhry N, Petry F. (2013) Innate immune responses against *Cryptosporidium parvum* infection. *Parasite Immunology*, 35 (2):55-64.

Korbel DS, Barakat FM, Di Santo JP, McDonald V.(2011). CD4+ T cells are not essential for control of early acute *Cryptosporidium parvum* infection in neonatal mice. *Infection and Immunity*. ;79(4):1647-53.

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