THE 5'-ECTOENZYME CD73 PROMOTES *TOXOPLASMA GONDII* PERSISTENCE IN THE CNS WHILE LIMITING SYSTEMIC IMMUNOPATHOLOGY

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Deeqa Abdishire Mahamed

January 2013

© 2013 Deeqa Abdishire Mahamed

THE 5'-ECTOENZYME CD73 PROMOTES *TOXOPLASMA GONDII* PERSISTENCE IN THE CNS WHILE LIMITING SYSTEMIC IMMUNOPATHOLOGY

Deeqa Abdishire Mahamed, Ph. D.

Cornell University 2013

The protozoan pathogen *Toxoplasma gondii* is a highly successful parasite that infects up to a third of the world's population, causing morbidity and mortality in the immunocompromised and when acquired congenitally. As an obligate intracellular pathogen, *T. gondii* has adapted to acquiring key nutrients from its host. Unlike vertebrate cells which are capable of *de novo* adenosine synthesis, *T. gondii* must rely solely on the purine salvage pathway, necessitating the presence of host-generated adenosine. CD73, present on vertebrate host cells but not *T. gondii* parasites, is a surface-anchored glycoprotein that catalyzes the conversion of AMP to adenosine, which is then sensed by the cell through transmembrane adenosine receptors. The enzyme is highly expressed in various tissues, including the brain, lymphoid organs, and in many immune cell subsets.

To determine the role of CD73-generated adenosine in *T. gondii* infection, I infected wildtype and CD73-knockout mice with the *Toxoplasma gondii* ME49 strain via the oral route. $CD73^{-/-}$ mice did not succumb to reactivation of infection, and had fewer brain cysts developing in the $CD73^{-/-}$ mice. The reduced cyst burden was due to a defect in *T. gondii* differentiation to cyst-forming bradyzoites in the brain in the absence of CD73, and was independent of adenosine receptor signaling. *In vitro* differentiation in primary murine astrocytes and human fibroblasts was also CD73-dependent, and could be rescued by exogenous supplementation with adenosine or blocked with pharmacological inhibition of CD73, while treatment with an adenosine receptor agonist had no effect. Thus CD73-

generated adenosine directly promoted *T. gondii* persistence and differentiation to long-lived tissue cysts.

To further investigate the reason for the reduced parasite burden in the CNS, I inoculated WT and CD73$^{-/-}$ mice with *T. gondii* via peritoneal inoculation. Unexpectedly, CD73$^{-/-}$ mice were markedly susceptible to *T. gondii* intraperitoneal infection. Susceptibility was associated with elevated IL1β, TNFα, IFNγ and nitric oxide production, and increased infiltration of neutrophils and T cells into the peritoneal cavity. CD73 expression on both hematopoietic and nonhematopoietic cells was required to prevent immunopathology, and the absence of CD73 promoted local dissemination of the parasite. Thus, CD73 plays opposite functions in acute and chronic infections with a protozoan pathogen.

BIOGRAPHICAL SKETCH

Deeqa Mahamed was born in Galkaio, Somalia. She completed secondary school at Sir Frederick Banting Secondary School in London Ontario, and received a bachelor of science at the University of Western Ontario, also in London, Ontario. Deeqa joined the PhD program in the Graduate Field of Immunology in 2007.

To my mother Fadumo Abdirahman Ahmed and all my friends and family.

ACKNOWLEDGMENTS

I would first like to thank Margaret Bynoe for her enthusiastic support, guidance and willingness to serve as my thesis advisor and lifelong mentor. The other members of my thesis committee Eric Denkers, Avery August and Susana Mendez gave helpful suggestions and indispensable advice and feedback. Dr. Craig Altier kindly served as my external examiner. My colleagues in the lab—Leah Alabanza, Jeff Mills, Dogeun Kim, Antje Krenz, Cindy Mueller and Nyki D'Elia—were generous with their time and assisted with many experiments. Present and past members of the Denkers lab—particularly Charlotte Egan, Delbert Abi Abdallah, Barbara Butcher, Sara Cohen, and Anne Schneider-- helped with many experiments and offered helpful suggestions and critiques.

Within the Department of Microbiology and Immunology, I would like to thank everyone for their feedback and support, with special acknowledge of Mary Linton, Sachiko Funaba, Karen Hollenbeck and Walt Iddings; who helped with many of the administrative necessities of graduate school throughout these years. Without the animal care and veterinary staff of the Transgenic Mouse Core Facility and the ECRF none of the experiments in this dissertation would have been possible.

Finally, I would like to thank my family and friends for their enthusiastic support and generosity. Their contributions to my life cannot be overstated or enumerated.

TABLE OF CONTENTS

Biographical Sketch ..... **iii**

Dedication ..... **iv**

Acknowledgements ..... **v**

CHAPTER 1 Introduction **1**

Significance 1

Toxoplasma gondii 1

General biology and life cycle 1

Innate immunity to T. gondii. 3

The adaptive immune response to Toxoplasma gondii 4

Modulation of the immune response by T. gondii 5

Toxoplasma gondii purine salvage 6

Toxoplasma gondii stage differentiation 7

CD73 and Extracellular Adenosine 8

CD73 (Ecto 5’-Nucleotidase) 8

Extracellular Adenosine 10

Extracellular adenosine in innate immunity and inflammation 11

Purinergic Signaling and Neutrophils 11

Purinergic Signaling in DCs and Macrophages. 11

Extracellular adenosine and T cell mediated immunity 12

Synthesis 14

References 15

CHAPTER 2 CD73-Generated Adenosine Facilitates Toxoplasma gondii Differentiation to Long-Lived Tissue Cysts in the Central Nervous System **27**

Abstract 28

Introduction 29

Materials and Methods 31

Mouse strains and in vivo infection 31

Parasite strains and maintenance 31
Histology 32
Flow cytometry 32
Quantitation of Gene Expression by qPCR 32
Quantitation of T. gondii by qPCR 33
Bradyzoite isolation 33
T. gondii proliferation 33
Astrocyte isolation and culture: 34
Dexamethasone Treatment 35
T. gondii differentiation and immunofluorescence microscopy 35
Data analysis 36
Results 36
$CD73^{-/-}$ mice are less susceptible to toxoplasmosis 36
$CD73^{-/-}$ and WT mice show similar levels of T. gondii parasite burden in the brain during the acute but not chronic stages of infection. 38
$CD73^{-/-}$ and WT mice have a similar degree of leukocyte infiltration into the brain during T. gondii chronic infection. 40
Brains from T. gondii infected $CD73^{-/-}$ and WT mice have similar immune effector gene expression kinetics. 41
Expression of key adhesion molecules and chemokines in the CNS during T. gondii infection. 42
Lack of CD73 enhances proinflammatory cytokine responses to T. gondii. 43
Infection and proliferation of T. gondii in astrocytes is not dependent on CD73 expression 45
CD73 expression promotes bradyzoite/cyst persistence in the brain 46
CD73 promotes tachyzoite to bradyzoite differentiation in astrocytes 47
Exogenous adenosine rescues T. gondii cyst formation in CD73-deficient host cells 50
Discussion 54
References 57

CHAPTER 3 CD73-generated adenosine is critical for immune regulation during Toxoplasma gondii infection **64**
Abstract 65
Introduction 66
Materials and Methods 67
Mice 67
Toxoplasma gondii infection 67
Histology 67
Treatment with adenosine receptor agonists and antagonists 67
Isolation of Peritoneal exudate cells 68
Isolation of spleen and mesenteric lymph node cells 68
Ex vivo Flow cytometry 68
Cytospins 69
Cell culture 69
Cytokine ELISAs 69
Measurement of nitrite production 69
Bone Marrow Chimeras 70
Adoptive transfer 70
Results 70
Intraperitoneal infection with Toxoplasma gondii is lethal in $CD73^{-/-}$ hosts 70
Treating $CD73^{-/-}$ mice with the adenosine receptor agonist NECA ablates susceptibility 72
The A_{2A} adenosine receptor knockout mouse recapitulates the susceptibility to ip infection observed in CD73-deficient hosts 73
Hematopoietic and nonhematopoietic cells contribute CD73-generated adenosine to limit inflammation 75
Susceptibility is associated with increased and sustained infiltration of neutrophils and T lymphocytes 77
Parasite control in CD73-deficient mice is limited 79
Increased inflammatory mediators in the absence of extracellular adenosine 81

CD73$^{-/-}$ retain a moderate resistance to chronic T. gondii infection despite early acute susceptibility 84
Discussion 85
References 86
CHAPTER 4 Summary and Conclusions 88
References 97
APPENDIX A 100

LIST OF FIGURES

CHAPTER 1 .... **1**

FIGURE 1.1 Current model of T. gondii purine salvage 7

FIGURE 1.2 CD73 and adenosine receptor signaling in inflammation 9

CHAPTER 2 .... **27**

FIGURE 2.1 CD73$^{-/-}$ mice are resistant to chronic T. gondii infection 37

FIGURE 2.2 CD73$^{-/-}$ mice have reduced parasite burden in the brain during chronic T. gondii infection 39

FIGURE 2.3 Kinetics of the immune response to T. gondii infection in the brain 40

FIGURE 2.4 Kinetics of MadCAM1, VCAM1 and CX3CL1 expression 43

FIGURE 2.5 Proinflammatory cytokine production 44

FIGURE 2.6 Proliferation of T. gondii in cultured astrocytes 46

FIGURE 2.7 Gene expression profile of T. gondii tachyzoites and bradyzoites 48

FIGURE 2.8. Differentiation of T. gondii in cultured astrocytes 51

FIGURE 2.9. Exogenous adenosine rescues T. gondii cyst formation 53

CHAPTER 3 .... **64**

FIGURE 3.1 CD73$^{-/-}$ mice are more susceptible to acute toxoplasmosis 71

FIGURE 3.2. Intraperitoneal infection with Toxoplasma gondii RH is more lethal in CD73$^{-/-}$ hosts 72

FIGURE 3.3. CD73-generated adenosine protects mice against inflammation via adenosine receptor signaling 73

FIGURE 3. 3.4. The A2A adenosine receptor mediates the protective effects of extracellular adenosine 74

FIGURE 3.5. Constitutive CD73 expression on resident PECs 75

FIGURE 3.6. Hematopoietic and nonhematopoietic cells contribute CD73-generated adenosine to limit inflammation 76

FIGURE 3.7. Absence of CD73 in the T or B cell compartment does not confer susceptibility to T. gondii 77

FIGURE 3.8. Cell composition comparison of peritoneal exudate cells (PECs) 78

FIGURE 3.9. Parasite dissemination during acute infection 80

FIGURE 3.10. Dysregulated cytokine responses in CD73$^{-/-}$ mice 82

FIGURE 3.11. Reduced IL-12 secretion by PECs from CD73$^{-/-}$ mice 83

FIGURE 3.12. Increased neutrophil recruitment into the peritoneal exudate cell compartment in CD73$^{-/-}$ mice 83

CHAPTER 4.. 88

FIGURE 4.1. 4.1. Proposed model of how CD73 may contribute to T. gondii purine salvage in an infected cell 92

APPENDIX A.. 100

FIGURE A1. . Proliferation of T. gondii bradyzoites isolated from wildtype and CD73$^{-/-}$ hosts 102

FIGURE A2. Dexamethasone induced recrudescence of chronic T. gondii infection 103

LIST OF TABLES

APPENDIX A.. 100

TABLE 1. Murine and T. gondii primer sequences used in real-time PCR 101

CHAPTER 1

Introduction

Significance

Toxoplasma gondii is a significant human pathogen, infecting up to one third of the world population. It is also of significance in veterinary medicine, since it is a zoonotic infection. Current effective treatments for *T. gondii* infection are limited, and thus novel therapies and insights into *T. gondii* virulence offer potential benefits. In addition, *T. gondii* biology offers insight into related organisms, especially apicomplexans and other intracellular protozoan parasites.

Toxoplasma gondii

General biology and life cycle

Toxoplasma gondii is an obligate intracellular protozoan pathogen with a broad host range and tissue tropism. *T. gondii* traffics to the central nervous system following invasion and replication in the gut. In healthy individuals, the parasite forms tissue cysts which limit parasite replication but enable the parasite and host to avoid immune cell-mediated destruction and tissue inflammation. Latent infection is common in healthy people, with prevalence estimated from 20 to 80% depending on country. Immunocompromised individuals can experience reactivation of infection, leading to toxoplasmic encephalitis. Unusual among the apicomplexa, *T. gondii* can complete its asexual cycle within intermediate hosts (any warm-blooded animal) without needing to undergo sexual division in a vector or in the definitive host (felids). This allows the parasite to be transmitted between intermediate hosts, likely contributing to the great success and clonal expansion of this parasite (1). *T. gondii* transmission is through ingestion of cysts or oocysts in contaminated meat or produce. The cysts and oocysts rupture in the gastrointestinal tract (from enzymatic digestion and in response to low pH), liberating infective bradyzoites or sporozoites respectively. These zoites transform to tachyzoites, which rapidly invade epithelial cells lining

the small intestine (mainly the ileum). Tachyzoites also invade immune cells in the lamina propria of the intestine, and dissemination of the parasite throughout the host is thought to be mediated by these infected immune cells(2, 3). Transport of live parasites to the central nervous system and skeletal muscle is mainly attributed to cells of the myeloid lineage, In the central nervous system, *T. gondii* invades neurons, astrocytes and microglia in which it establishes a chronic infection by differentiating to form long-lived tissue cysts (4, 5).

In North America and Europe the most common *T. gondii* strains are classified into three clonal lineages (types I, II, and III), although strains not belonging to these lineages are being increasingly isolated, particularly in wildlife in North America and in South America, Australia and Africa (6-10). Type I strains, such as RH and GT1, are highly virulent in rodents, and an infectious dose of one viable parasite can be lethal in one to two weeks (11). They are usually associated with a worse prognosis in humans. Type II strains (ME49, PTG) are of intermediate virulence, and can be sustained by rodent hosts long term. Type III strains (CTG) are avirulent in rodents.

Innate immunity to T. gondii

Many components and cells of the innate immune system are critical for control of *T. gondii*, both in direct killing of parasites, as well as in induction of the adaptive immune response (12-15). Experimental depletion of T cells (16), B cells (17) and neutrophils (18) leads to increased susceptibility in mice. Genetic knockouts of innate molecules and cytokines have elucidated the important roles of the adapter protein MyD88, the cytokines IL-12 (19) and IL-18, pattern recognition receptors (particularly toll-like receptors 2, 4, and 11) nitric oxide production via inducible nitric oxide synthase (iNOS), as well as other intracellular immune mediators such as IGTPases (20). Toll-like receptor recognition of *T. gondii* is mediated through TLR2, -4, and

the recently identified TLR11, which is active in rodents but nonfunctional in humans. TLR11 binds to profilin, a parasite protein implicated in gliding motility, invasion and host cell egress TLR11-deficient mice show increased susceptibility to *T. gondii* infection(12, 21). Dendritic cells are the main cell type that recognizes *T. gondii* via TLR11 and initiates the immune response through the release of IL-12, although neutrophils and macrophages are also important sources of IL-12 (18, 22). NK cells are an early and important source of interferon gamma (23). The intracellular stages of the *T. gondii* lifecycle are required for parasite replication, and also help the parasite evade neutralization by the humoral immune system, but requires specific adaptations to survive. Thus while *T. gondii* induces a potent immune response, it also modulates key factors.

The adaptive immune response to T. gondii

T. gondii induces a robust T_h1 response which protects against unchecked parasite growth both in the initial phase of infection as well as during latent infection. Although *T. gondii* tachyzoites disseminate to many distant organs, including lungs, liver, lymph nodes, spleen and heart, the parasite persists mainly in brain and skeletal muscle through formation of tissue cysts. Formation of cysts requires parasite differentiation to bradyzoites. Bradyzoites are less immunogenic than tachyzoites, which likely promotes survival of both parasite and host. The initial *T. gondii* CNS infection induces robust recruitment of immune cells, especially T cells and macrophages. These cells are known to be necessary for the control of *T. gondii* proliferation, as demonstrated by various mouse models in which T cells or innate immune mediators are compromised (16). Control of parasite replication involves a variety of mechanisms, including killing of infected cells by cytotoxic T lymphocytes(24), starving the parasite of nutrients, and

release of inflammatory mediators (25). The cytokine interferon gamma from $CD4^+$ T cells and $CD8^+$ T cells controls tachyzoite replication and promotes parasite killing by macrophages . In addition to its importance during acute infection, IFNγ is also critical for control of *T. gondii* during latent infection, as ablation of this cytokine leads to fatal toxoplasmic encephalitis in chronically infected mice (26). Nevertheless, IFNγ and various immune cell subsets have also been associated with immunopathology in susceptible hosts (27, 28), especially in response to highly virulent *T. gondii* strains (29)

Similar to the periphery, the immune response to *T. gondii* in the CNS is dominated by IFNγ produced by $CD4^+$ and $CD8^+$ T cells. Depletion of IFNγ after the establishment of chronic infection leads to rapid recrudescence of *T. gondii* cysts, differentiation of bradyzoites to host cell-lytic tachyzoites, and is ultimately lethal. The cytotoxic T cell response is also important during chronic infection, as evidenced by infection of $CD8^+$ T cell depleted or perforin deficient murine hosts (30, 31).

Modulation of the immune response by T. gondii

As a highly successful pathogen, *T. gondii* modulates the immune response extensively. To disseminate from the initial site of infection, the parasite may induce the migration of infected dendritic cells and monocytes/macrophages (32). The parasite also inhibits macrophage, neutrophil and dendritic cell cytokine release or activation (33, 34). Although the modulation of the immune response protects the parasite from the immune system, it also protects the host from pathological inflammation, and genetic susceptibility to lethal infection with less virulent strains of *T. gondii* may represent over-production of inflammatory cytokines rather than a failure to control parasite replication (27, 29, 35). The absence of regulatory factors such as IL-10 is lethal

for infected hosts, again underscoring the importance of a balanced immune response that inhibits parasite growth without inducing lethal immunopathology (36).

***T. gondii* purine salvage**

T. gondii, like other apicomplexans, has evolved a strictly parasitic niche and consequently lost the ability to synthesize the purine ring *de novo*. Instead, the parasite salvages host adenosine through a parasite membrane transporter and two redundant intracellular salvage enzymes, adenosine kinase (AK) and hypoxanthine-xanthine-guanine-phosphoribosyltransferase (HXGPRT) (37) (Figure 1.1). Although neither enzyme is critical for parasite survival individually, double-knockout of both adenosine kinase and HXGPRT is lethal for *T. gondii* (37-39), demonstrating the critical nature of the purine salvage pathway for parasite survival and replication. Interestingly, while *T. gondii* also possesses a highly active and abundant nucleotide triphosphate hydrolase (NTPDase), which can convert adenosine triphosphate sequentially to ADP and then AMP) (40), in addition to a dedicated adenosine transporter (41, 42), it lacks a 5'-nucleotidase that can further convert AMP to adenosine, whereas host cells have both extracellular and intracellular 5'-nucleotidases in addition to the ability to synthesize purines *de novo*. Therefore the purine salvage pathway offers a promising target of drug development.

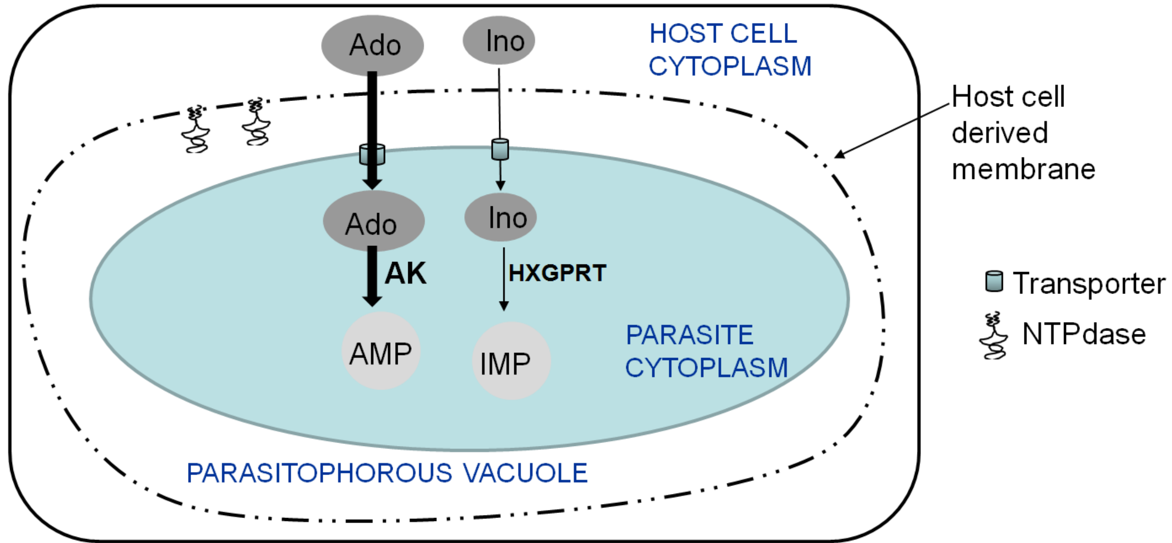


Figure 1.1. Current model of *T. gondii* purine salvage. Purines are salvaged from the cytoplasm of the host cell as adenosine (Ado) or inosine (Ino) via transporters on the parasite plasma membrane. Within the parasite cytoplasm, Ado and Ino are phosphorylated to AMP and IMP by the enzymes adenosine kinase (AK) and hypoxanthine-xanthine-guanine-phosphoribosyltransferase (HXGPRT), respectively. The *T. gondii* nucleoside triphosphate diphosphohydrolase (NTPDase) is found in the parasitophorous vacuole (PV) and the PV membrane. Size of the arrows represents the contribution of each pathway to purine salvage in *T. gondii*.

T. gondii stage differentiation

T. gondii undergoes four distinct developmental stages, two of which develop strictly in the parasite's definite hosts, felids. All other warm-blooded animals can also harbor tachyzoites and bradyzoites, which replicate by asexual division known as endodyogeny. After ingestion of tissue cysts or oocysts, bradyzoites are liberated in the small intestine, where they invade epithelial cells and differentiate into tachyzoites. Tachyzoites divide rapidly and disseminate

throughout the host. Parasitized immune cells are thought to transport the tachyzoites to the brain and skeletal muscle, a site of further *T. gondii* differentiation(5, 32). In these tissues, a combination of host and parasite signals induce tachyzoite-to-bradyzoite conversion, leading to the formation of tissue cysts and the persistence of the parasite as a latent infection (43).

This stage differentiation is critical for parasitism for two reasons. First, tissue cysts can be ingested by susceptible hosts, allowing for successful transmission of the parasite to naïve hosts. Secondly, the cyst stage allows the parasite to remain dormant for the life of the host, sequestering bradyzoites from the immune response in the immunologically privileged central nervous system. Indeed, in adult humans, recrudescence of dormant infection following immune system inhibition (such as in AIDS or following cancer chemotherapy) remains the major disease risk. Therefore understanding the mechanisms by which *T. gondii* persists in the CNS could enable timely therapeutic interventions for the immunocompromised.

CD73 and Extracellular Adenosine

CD73 (Ecto 5'-Nucleotidase)

CD73, also known as ecto-5'-nucleotidase, is a 70 kDa cell surface glycoprotein that catalyzes the conversion of AMP to adenosine in the extracellular and pericellular space. CD73 is highly conserved in primates, while mouse and human CD73 share 88% homology; chicken, zebrafish, and fruit flies also have CD73 homologs. CD73 is anchored to the plasma membrane as a homodimer through a glycophosphatidylinositol (GPI) linker covalently attached to its C-terminus during posttranslational processing. This attachment moiety may impart unique properties on proteins, including association with or enrichment of these proteins in lipid rafts. In the extracellular milieu, adenosine serves as a potent immune modulator through binding to adenosine receptors (Figure. 1.2). CD73 itself has been shown to function as a T cell

costimulatory receptor (44, 45), and has also been proposed to act as an adhesion molecule on T cells, with an as yet unknown ligand on endothelial and epithelial cells promoting cell-cell attachment and T cell extravasation (46-49). At the same time, CD73 expressed on endothelial cells has been shown to be involved in limiting T cell extravasation by downregulating adhesion molecules (50). In contrast, our lab has shown that epithelial cells in the choroid plexus mediate T cell infiltration of the CNS in experimental autoimmune encephalitis in a mechanism involving signaling through adenosine receptors (51). This interesting dichotomy may play a critical role in the immune response to *T. gondii* infection, especially during the acute phase of infection.

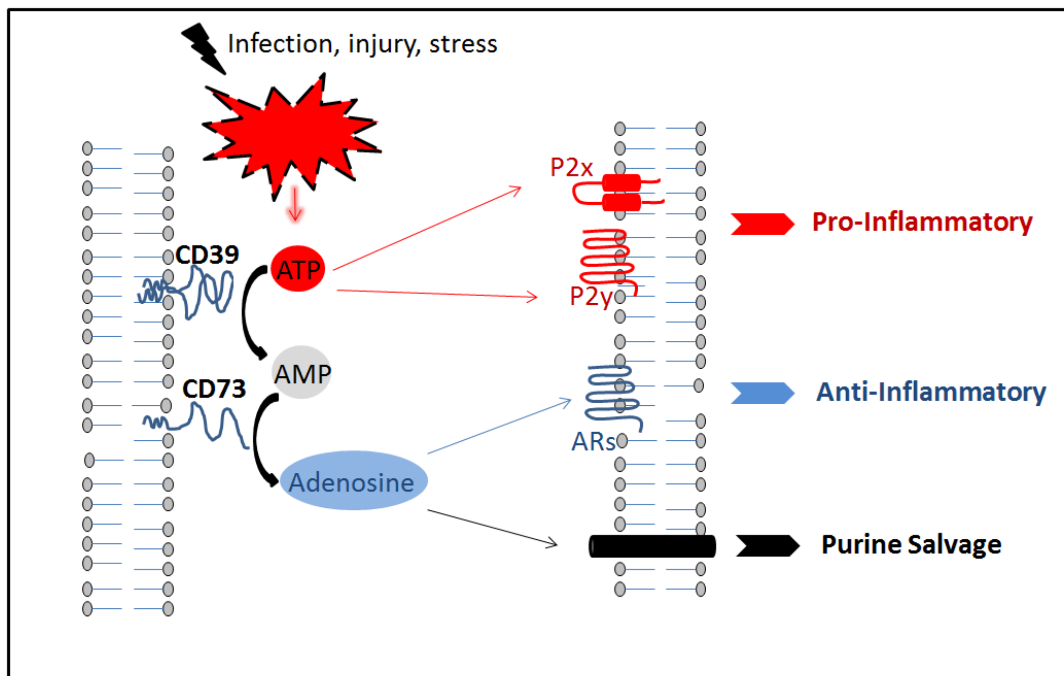


Figure 1.2. CD73 and adenosine receptor signaling in inflammation. Cell death or damage releases ATP into the extracellular milieu, which acts as a "danger signal" by binding to P2X and P2Y receptors and mediating immune cell recruitment, activation and function. This cascade must be resolved by the concerted actions of CD39 and CD73, which break down ATP to ADP, AMP and finally adenosine. Adenosine is a ligand for P1 receptors, which largely mediate anti-

inflammatory responses. Adenosine can also be taken up by adenosine transporters for intracellular salvage.

Extracellular Adenosine

ATP is released during tissue injury, infection and in inflammation and acts as a danger signal in the extracellular matrix, synergizing with other mediators (such as cytokines, TLR ligands, and specific antigens) to promote activation and migration of innate and adaptive immune cells through binding to P2X and P2Y receptors (52-55). At the resolution of inflammation, ATP's potent effect is neutralized through extracellular enzymes that break down the nucleotide. CD39 and CD73 sequentially convert extracellular ATP to ADP, AMP and then to adenosine. CD39 and CD73 have been implicated in immune system regulation (56). Adenosine itself modulates the inflammatory response by binding to a group of four G-coupled protein receptors known as P1 adenosine receptors. A_{2A} and A_{2B} receptors are linked to G_s proteins which activate adenyl cyclase and thus elevate intracellular levels of cAMP, while A1 and A3 are linked to G_i proteins and inhibit cAMP generation. Downstream of A_{2A}/A_{2B} induced cAMP, the pleiotropic enzyme PKA interacts with various signaling cascades (ERK1/2, CREB) that lead to gene regulation or activation and cytoskeletal network rearrangement, to ultimately effect a shift in cell activation or responsiveness. A_1 and A_3 signaling activates phospholipase C through the $G_{\beta\gamma}$ subunits, which induces MAPK cascade and also feeds into growth and differentiation pathways. The outcome of adenosine receptor signaling is based on cellular target and the microenvironment. Interestingly, production of extracellular adenosine is often a multicellular process, with CD39 on neutrophils or regulatory T cells breaking down ATP to AMP, which may be further catabolized to adenosine by CD73-expressing platelets in the vicinity. The resulting molecule can target nearby platelets, neutrophils, T cells or endothelial

and epithelial cells. However, adenosine's effects are highly localized, as it can be rapidly degraded to inosine by adenosine deaminase (ADA) or internalized by adenosine transporters.

Extracellular adenosine in innate immunity and inflammation

In general, the outcome of adenosine receptor signaling in innate immune cells is anti-inflammatory. However, a number of reports suggest that adenosine receptor signaling may also enhance or complement proinflammatory processes, such as in IL-6 and type I interferon production (57-60). On the other hand, endogenous purine nucleoside signaling can also promote diseases such as cancer. Tumor immune evasion through the release of T cell-inhibitory adenosine levels has been demonstrated using various murine and human models of cancer (61-63).

Purinergic Signaling and Neutrophils

Indirectly, adenosine also antagonizes the expression of adhesion molecules on endothelial cells. In addition to limiting accumulation via endothelial cells, adenosine has potent effects on neutrophils themselves, including inhibition of cytokine release and generation of the respiratory burst, inhibition of cellular adhesion to the ECM and to other cells, and ultimately induction of apoptosis (64-69). On neutrophils, A_{2A} adenosine receptor signaling inhibits extravasation and adhesion, limiting neutrophil accumulation at the site of inflammation (70-73). Since neutrophils are also involved in the generation of extracellular adenosine (74), they are well situated to regulating their own activities as well as that of neighboring cells.

Purinergic Signaling in DCs and Macrophages.

Dendritic cells and macrophages (including microglia, Langerhans cells, Kupfer cells and other tissue-resident myeloid-derived cells) express varying levels of all four adenosine receptors

and some subsets also express CD73 (75, 76). Thus these cells can respond to and potentially generate extracellular adenosine. Adenosine receptor signaling in dendritic cells generally inhibits production of pro-inflammatory cytokines and chemokines and tissue remodeling factors such as TNFα (77-79) and IL1β (80), MMP9 (81, 82). As professional antigen presenting cells, DCs are well-situated to promote or thwart cell-mediated immune responses, and to skew said immune response towards a T_h1 or T_h2 pathway(83). The response of DCs to adenosine receptor stimulation depends partially on their maturation state and subset (84, 85). Schnurr *et al*. report a differentiation stage difference in the response of plasmacytoid DCs to adenosine. Immature DCs expressed mainly A_1AR and were activated to release cytokines and migrate in response to adenosine, whereas mature DCs downregulated A_1AR, upregulated A_{2AAR}, and responded to adenosine by downregulating cytokine production(86). Monocyte-derived DCs respond to adenosine receptor signaling by priming T cells towards a T_h2 phenotype (87).

Macrophages produce TNFα and IL-12 in response to LPS challenge, a process antagonized by A2A adenosine receptor stimulation (88-91). At the same time, LPS-induced IL-10 production is enhanced (92, 93), so that the two pathways synergistically downregulate proinflammatory factors. Likewise, microglia have been reported to produce IL-10 and downregulate LPS-induced TNFα in response to A2A, A2B and A3 signaling (94, 95). In general, macrophages shift from a classically activated to an alternatively activated phenotype in response to adenosine receptor ligation, which is consistent with the role of adenosine in promoting the resolution of inflammation and wound-healing.

Extracellular adenosine and T cell mediated immunity

Subsets of T cells express CD39 and CD73, and adenosine receptors. In the thymus, CD73 expression increases with T cell maturation. In lymphoid tissues, CD73 is highly

expressed on $CD8^+$ T cells. Among $CD4^+$ T cells, the highest expression of CD73 is found on T regulatory cells (T_{regs}), which also express. CD73- or CD39-deficient T_{regs} are impaired in regulation of effector T cells (56). T cells express A_{2A}. Signaling through A_{2A} leads to suppression of T effector functions, such as cytokine release, proliferation and accumulation at sites of inflammation(96). However, the effect of adenosine on T cell infiltration can be modulated by the local microenvironment. In experimental autoimmune encephalitis, the murine model for multiple sclerosis, CD73-generated adenosine enhances infiltration of pathogenic T cells into the CNS (51).

Synthesis

The results presented in this study describe the dual role of CD73-generated adenosine in *T. gondii* pathogenesis. In **Chapter 2**, I show that CD73 promotes *T. gondii* chronic infection through a mechanism independent of adenosine receptor signaling. Analysis of the immune response to *T. gondii* in the CD73-deficient murine host showed a slight delay in T cell infiltration into the CNS. Although initial parasite expansion and dissemination was unimpaired in $CD73^{-/-}$ mice, cyst formation or persistence was reduced significantly, and the parasite burden during chronic infection was subsequently lower in $CD73^{-/-}$ hosts. Further experiments determined that CD73 expression on host cells harboring bradyzoites was required for efficient *T. gondii* cyst formation. Since the adenosine receptor agonist NECA did not rescue cyst formation in $CD73^{-/-}$ astrocytes, and A1 and A2A receptor-knockout mice had a brain cyst burden similar to WT, I concluded that *T. gondii* purine salvage pathway involves the activity of CD73.

In **Chapter 3**, I describe the key regulatory role of extracellular adenosine in murine *T. gondii* infection. In contrast to the chronic infection model described in Chapter 2, here I describe experiments infecting $CD73^{-/-}$ mice by the systemic intraperitoneal route. I found a profound defect in immune regulation during the acute phase of infection, with $CD73^{-/-}$ mice exhibiting increased morbidity and mortality associated with an uncontrolled immune response to *T. gondii*. I found increased recruitment of neutrophils and T cells, combined with elevated levels of IL1β, TNFα, IFNγ and nitric oxide, induced by infection in the absence of adenosine receptor signaling. In **Chapter 4**, I summarize my results and present future directions aimed at addressing the many questions prompted by my findings.

References

1. Minot S, *et al.* (2012) Admixture and recombination among Toxoplasma gondii lineages explain global genome diversity. *Proc Natl Acad Sci U S A* 109(33):13458-13463.
2. Sumyuen MH, Garin YJ, & Derouin F (1995) Early kinetics of Toxoplasma gondii infection in mice infected orally with cysts of an avirulent strain. *The Journal of parasitology* 81(2):327-329.
3. Dubey JP (1997) Bradyzoite-induced murine toxoplasmosis: stage conversion, pathogenesis, and tissue cyst formation in mice fed bradyzoites of different strains of Toxoplasma gondii. *J Eukaryot Microbiol* 44(6):592-602.
4. Lambert H & Barragan A (2010) Modelling parasite dissemination: host cell subversion and immune evasion by Toxoplasma gondii. *Cell Microbiol* 12(3):292-300.
5. Courret N, *et al.* (2006) CD11c- and CD11b-expressing mouse leukocytes transport single Toxoplasma gondii tachyzoites to the brain. *Blood* 107(1):309-316.
6. Ferreira AM, Martins MS, & Vitor RW (2001) Virulence for BALB/c mice and antigenic diversity of eight Toxoplasma gondii strains isolated from animals and humans in Brazil. *Parasite* 8(2):99-105.
7. Mercier A, *et al.* (2010) Additional haplogroups of Toxoplasma gondii out of Africa: population structure and mouse-virulence of strains from Gabon. *PLoS Negl Trop Dis* 4(11):e876.
8. Dubey JP, *et al.* (2010) A new atypical highly mouse virulent Toxoplasma gondii genotype isolated from a wild black bear in Alaska. *J Parasitol* 96(4):713-716.
9. Parameswaran N, *et al.* (2010) Non-archetypal Type II-like and atypical strains of Toxoplasma gondii infecting marsupials of Australia. *Int J Parasitol* 40(6):635-640.

10. Dubey JP (1981) Isolation of encysted Toxoplasma gondii from musculature of moose and pronghorn in Montana. *Am J Vet Res* 42(1):126-127.

11. Mordue DG, Monroy F, La Regina M, Dinarello CA, & Sibley LD (2001) Acute toxoplasmosis leads to lethal overproduction of Th1 cytokines. *J Immunol* 167(8):4574-4584.

12. Yarovinsky F, Hieny S, & Sher A (2008) Recognition of Toxoplasma gondii by TLR11 prevents parasite-induced immunopathology. *J Immunol* 181(12):8478-8484.

13. Scanga CA*, et al.* (2002) Cutting edge: MyD88 is required for resistance to Toxoplasma gondii infection and regulates parasite-induced IL-12 production by dendritic cells. *J Immunol* 168(12):5997-6001.

14. Yarovinsky F & Sher A (2006) Toll-like receptor recognition of Toxoplasma gondii. *Int J Parasitol* 36(3):255-259.

15. Sukhumavasi W*, et al.* (2008) TLR adaptor MyD88 is essential for pathogen control during oral toxoplasma gondii infection but not adaptive immunity induced by a vaccine strain of the parasite. *J Immunol* 181(5):3464-3473.

16. Araujo FG (1991) Depletion of L3T4+ (CD4+) T lymphocytes prevents development of resistance to Toxoplasma gondii in mice. *Infection and immunity* 59(5):1614-1619.

17. Kang H, Remington JS, & Suzuki Y (2000) Decreased resistance of B cell-deficient mice to infection with Toxoplasma gondii despite unimpaired expression of IFN-gamma, TNF-alpha, and inducible nitric oxide synthase. *J Immunol* 164(5):2629-2634.

18. Bliss SK, Gavrilescu LC, Alcaraz A, & Denkers EY (2001) Neutrophil depletion during Toxoplasma gondii infection leads to impaired immunity and lethal systemic pathology. *Infect Immun* 69(8):4898-4905.

19. Gazzinelli RT*, et al.* (1994) Parasite-induced IL-12 stimulates early IFN-gamma synthesis and resistance during acute infection with Toxoplasma gondii. *J Immunol* 153(6):2533-2543.

20. Taylor GA, Feng CG, & Sher A (2004) p47 GTPases: regulators of immunity to intracellular pathogens. *Nat Rev Immunol* 4(2):100-109.

21. Yarovinsky F*, et al.* (2005) TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science* 308(5728):1626-1629.

22. Bliss SK, Marshall AJ, Zhang Y, & Denkers EY (1999) Human polymorphonuclear leukocytes produce IL-12, TNF-alpha, and the chemokines macrophage-inflammatory protein-1 alpha and -1 beta in response to Toxoplasma gondii antigens. *J Immunol* 162(12):7369-7375.

23. Denkers EY, Gazzinelli RT, Martin D, & Sher A (1993) Emergence of NK1.1+ cells as effectors of IFN-gamma dependent immunity to Toxoplasma gondii in MHC class I-deficient mice. *J Exp Med* 178(5):1465-1472.

24. Hakim FT*, et al.* (1991) CD8+ T cells from mice vaccinated against Toxoplasma gondii are cytotoxic for parasite-infected or antigen-pulsed host cells. *J Immunol* 147(7):2310-2316.

25. Suzuki Y*, et al.* (2010) Removal of Toxoplasma gondii cysts from the brain by perforin-mediated activity of CD8+ T cells. *Am J Pathol* 176(4):1607-1613.

26. Daubener W & Hadding U (1997) Cellular immune reactions directed against Toxoplasma gondii with special emphasis on the central nervous system. *Med Microbiol Immunol* 185(4):195-206.

27. Liesenfeld O, Kosek J, Remington JS, & Suzuki Y (1996) Association of CD4+ T cell-dependent, interferon-gamma-mediated necrosis of the small intestine with genetic susceptibility of mice to peroral infection with Toxoplasma gondii. *J Exp Med* 184(2):597-607.

28. Egan CE*, et al.* (2009) CCR2-dependent intraepithelial lymphocytes mediate inflammatory gut pathology during Toxoplasma gondii infection. *Mucosal Immunol* 2(6):527-535.

29. Gavrilescu LC & Denkers EY (2001) IFN-gamma overproduction and high level apoptosis are associated with high but not low virulence Toxoplasma gondii infection. *J Immunol* 167(2):902-909.

30. Denkers EY*, et al.* (1997) Perforin-mediated cytolysis plays a limited role in host resistance to Toxoplasma gondii. *J Immunol* 159(4):1903-1908.

31. Parker SJ, Roberts CW, & Alexander J (1991) CD8+ T cells are the major lymphocyte subpopulation involved in the protective immune response to Toxoplasma gondii in mice. *Clinical and experimental immunology* 84(2):207-212.

32. Bierly AL, Shufesky WJ, Sukhumavasi W, Morelli AE, & Denkers EY (2008) Dendritic cells expressing plasmacytoid marker PDCA-1 are Trojan horses during Toxoplasma gondii infection. *J Immunol* 181(12):8485-8491.

33. Bennouna S, Sukhumavasi W, & Denkers EY (2006) Toxoplasma gondii inhibits toll-like receptor 4 ligand-induced mobilization of intracellular tumor necrosis factor alpha to the surface of mouse peritoneal neutrophils. *Infect Immun* 74(7):4274-4281.

34. Denkers EY, Kim L, & Butcher BA (2003) In the belly of the beast: subversion of macrophage proinflammatory signalling cascades during Toxoplasma gondii infection. *Cell Microbiol* 5(2):75-83.

35. McLeod R*, et al.* (1989) Immune responses associated with early survival after peroral infection with Toxoplasma gondii. *Journal of immunology* 142(9):3247-3255.

36. Gazzinelli RT*, et al.* (1996) In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. *Journal of immunology* 157(2):798-805.

37. Chaudhary K*, et al.* (2004) Purine salvage pathways in the apicomplexan parasite Toxoplasma gondii. *J Biol Chem* 279(30):31221-31227.

38. el Kouni MH (2007) Adenosine metabolism in Toxoplasma gondii: potential targets for chemotherapy. *Curr Pharm Des* 13(6):581-597.

39. Yu L, Gao YF, Li X, Qiao ZP, & Shen JL (2009) Double-stranded RNA specific to adenosine kinase and hypoxanthine-xanthine-guanine-phosphoribosyltransferase retards growth of Toxoplasma gondii. *Parasitol Res* 104(2):377-383.

40. Asai T & Suzuki Y (1990) Remarkable activities of nucleoside triphosphate hydrolase in the tachyzoites of both virulent and avirulent strains of Toxoplasma gondii. *FEMS Microbiol Lett* 72(1-2):89-92.

41. Schwab JC, Afifi Afifi M, Pizzorno G, Handschumacher RE, & Joiner KA (1995) Toxoplasma gondii tachyzoites possess an unusual plasma membrane adenosine transporter. *Mol Biochem Parasitol* 70(1-2):59-69.

42. De Koning HP, Al-Salabi MI, Cohen AM, Coombs GH, & Wastling JM (2003) Identification and characterisation of high affinity nucleoside and nucleobase transporters in Toxoplasma gondii. *Int J Parasitol* 33(8):821-831.

43. Bohne W, Heesemann J, & Gross U (1994) Reduced replication of Toxoplasma gondii is necessary for induction of bradyzoite-specific antigens: a possible role for nitric oxide in triggering stage conversion. *Infect Immun* 62(5):1761-1767.

44. Resta R & Thompson LF (1997) T cell signalling through CD73. *Cell Signal* 9(2):131-139.

45. Massaia M*, et al.* (1991) Amplification of T cell activation induced by CD73 (ecto-5' nucleotidase) engagement. *Adv Exp Med Biol* 309B:155-158.

46. Colgan SP, Eltzschig HK, Eckle T, & Thompson LF (2006) Physiological roles for ecto-5'-nucleotidase (CD73). *Purinergic Signal* 2(2):351-360.

47. Volmer JB, Thompson LF, & Blackburn MR (2006) Ecto-5'-nucleotidase (CD73)-mediated adenosine production is tissue protective in a model of bleomycin-induced lung injury. *J Immunol* 176(7):4449-4458.

48. Arvilommi AM, Salmi M, Airas L, Kalimo K, & Jalkanen S (1997) CD73 mediates lymphocyte binding to vascular endothelium in inflamed human skin. *Eur J Immunol* 27(1):248-254.

49. Airas L*, et al.* (1995) CD73 is involved in lymphocyte binding to the endothelium: characterization of lymphocyte-vascular adhesion protein 2 identifies it as CD73. *J Exp Med* 182(5):1603-1608.

50. Jalkanen S & Salmi M (2008) VAP-1 and CD73, endothelial cell surface enzymes in leukocyte extravasation. *Arterioscler Thromb Vasc Biol* 28(1):18-26.

51. Mills JH, *et al.* (2008) CD73 is required for efficient entry of lymphocytes into the central nervous system during experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* 105(27):9325-9330.

52. Miller CM, *et al.* (2011) The role of the P2X(7) receptor in infectious diseases. *PLoS Pathog* 7(11):e1002212.

53. Kawamura H, Kawamura T, Kanda Y, Kobayashi T, & Abo T (2012) Extracellular ATP-stimulated macrophages produce macrophage inflammatory protein-2 which is important for neutrophil migration. *Immunology* 136(4):448-458.

54. Di Virgilio F (2007) Liaisons dangereuses: P2X(7) and the inflammasome. *Trends Pharmacol Sci* 28(9):465-472.

55. Franchi L, Kanneganti TD, Dubyak GR, & Nunez G (2007) Differential requirement of P2X7 receptor and intracellular K+ for caspase-1 activation induced by intracellular and extracellular bacteria. *J Biol Chem* 282(26):18810-18818.

56. Deaglio S, *et al.* (2007) Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med* 204(6):1257-1265.

57. Sun Y, Wu F, Sun F, & Huang P (2008) Adenosine promotes IL-6 release in airway epithelia. *J Immunol* 180(6):4173-4181.

58. Sitaraman SV, *et al.* (2001) Neutrophil-epithelial crosstalk at the intestinal lumenal surface mediated by reciprocal secretion of adenosine and IL-6. *J Clin Invest* 107(7):861-869.

59. Vazquez JF, Clement HW, Sommer O, Schulz E, & van Calker D (2008) Local stimulation of the adenosine A2B receptors induces an increased release of IL-6 in mouse striatum: an in vivo microdialysis study. *J Neurochem* 105(3):904-909.

60. Wilson JM, *et al.* (2011) The A2B adenosine receptor promotes Th17 differentiation via stimulation of dendritic cell IL-6. *J Immunol* 186(12):6746-6752.

61. Jin D, *et al.* (2010) CD73 on tumor cells impairs antitumor T-cell responses: a novel mechanism of tumor-induced immune suppression. *Cancer Res* 70(6):2245-2255.

62. Zhang B (2012) CD73 promotes tumor growth and metastasis. *Oncoimmunology* 1(1):67-70.

63. Hausler SF, *et al.* (2011) Ectonucleotidases CD39 and CD73 on OvCA cells are potent adenosine-generating enzymes responsible for adenosine receptor 2A-dependent suppression of T cell function and NK cell cytotoxicity. *Cancer Immunol Immunother* 60(10):1405-1418.

64. Grenz A, *et al.* (2012) Adora2b Adenosine Receptor Signaling Protects during Acute Kidney Injury via Inhibition of Neutrophil-Dependent TNF-alpha Release. *J Immunol*.

65. Sroussi HY, Lu Y, Zhang QL, Villines D, & Marucha PT (2010) S100A8 and S100A9 inhibit neutrophil oxidative metabolism in-vitro: involvement of adenosine metabolites. *Free Radic Res* 44(4):389-396.

66. Cronstein BN, Kramer SB, Weissmann G, & Hirschhorn R (1983) Adenosine: a physiological modulator of superoxide anion generation by human neutrophils. *J Exp Med* 158(4):1160-1177.

67. Cronstein BN, Kubersky SM, Weissmann G, & Hirschhorn R (1987) Engagement of adenosine receptors inhibits hydrogen peroxide (H2O2-) release by activated human neutrophils. *Clin Immunol Immunopathol* 42(1):76-85.

68. Dianzani C, Brunelleschi S, Viano I, & Fantozzi R (1994) Adenosine modulation of primed human neutrophils. *Eur J Pharmacol* 263(1-2):223-226.

69. Roberts PA, Newby AC, Hallett MB, & Campbell AK (1985) Inhibition by adenosine of reactive oxygen metabolite production by human polymorphonuclear leucocytes. *Biochem J* 227(2):669-674.

70. Okusa MD*, et al.* (2000) A(2A) adenosine receptor-mediated inhibition of renal injury and neutrophil adhesion. *Am J Physiol Renal Physiol* 279(5):F809-818.

71. Hasko G & Cronstein BN (2004) Adenosine: an endogenous regulator of innate immunity. *Trends Immunol* 25(1):33-39.

72. Reutershan J*, et al.* (2009) Adenosine and inflammation: CD39 and CD73 are critical mediators in LPS-induced PMN trafficking into the lungs. *FASEB J* 23(2):473-482.

73. Felsch A, Stocker K, & Borchard U (1995) Phorbol ester-stimulated adherence of neutrophils to endothelial cells is reduced by adenosine A2 receptor agonists. *J Immunol* 155(1):333-338.

74. Eltzschig HK*, et al.* (2006) ATP release from activated neutrophils occurs via connexin 43 and modulates adenosine-dependent endothelial cell function. *Circ Res* 99(10):1100-1108.

75. Hammarberg C, Schulte G, & Fredholm BB (2003) Evidence for functional adenosine A3 receptors in microglia cells. *J Neurochem* 86(4):1051-1054.

76. van der Putten C*, et al.* (2009) Differential expression of adenosine A3 receptors controls adenosine A2A receptor-mediated inhibition of TLR responses in microglia. *J Immunol* 182(12):7603-7612.

77. Kreckler LM, Wan TC, Ge ZD, & Auchampach JA (2006) Adenosine inhibits tumor necrosis factor-alpha release from mouse peritoneal macrophages via A2A and A2B but not the A3 adenosine receptor. *J Pharmacol Exp Ther* 317(1):172-180.

78. Fotheringham JA, Mayne MB, Grant JA, & Geiger JD (2004) Activation of adenosine receptors inhibits tumor necrosis factor-alpha release by decreasing TNF-alpha mRNA stability and p38 activity. *Eur J Pharmacol* 497(1):87-95.

79. Martin L, Pingle SC, Hallam DM, Rybak LP, & Ramkumar V (2006) Activation of the adenosine A3 receptor in RAW 264.7 cells inhibits lipopolysaccharide-stimulated tumor necrosis factor-alpha release by reducing calcium-dependent activation of nuclear factor-kappaB and extracellular signal-regulated kinase 1/2. *J Pharmacol Exp Ther* 316(1):71-78.

80. Sipka S*, et al.* (2005) Adenosine inhibits the release of interleukin-1beta in activated human peripheral mononuclear cells. *Cytokine* 31(4):258-263.

81. Zhao P*, et al.* (2008) Hypoxia suppresses the production of MMP-9 by human monocyte-derived dendritic cells and requires activation of adenosine receptor A2b via cAMP/PKA signaling pathway. *Mol Immunol* 45(8):2187-2195.

82. Chen H*, et al.* (2011) Regulation of MMP-9 expression by the A2b adenosine receptor and its dependency on TNF-alpha signaling. *Exp Hematol* 39(5):525-530.

83. Cobbold SP, Adams E, Nolan KF, Regateiro FS, & Waldmann H (2010) Connecting the mechanisms of T-cell regulation: dendritic cells as the missing link. *Immunol Rev* 236:203-218.

84. Idzko M*, et al.* (2002) Nucleotides induce chemotaxis and actin polymerization in immature but not mature human dendritic cells via activation of pertussis toxin-sensitive P2y receptors. *Blood* 100(3):925-932.

85. Shin A*, et al.* (2008) P2Y receptor signaling regulates phenotype and IFN-alpha secretion of human plasmacytoid dendritic cells. *Blood* 111(6):3062-3069.

86. Schnurr M, *et al.* (2004) Role of adenosine receptors in regulating chemotaxis and cytokine production of plasmacytoid dendritic cells. *Blood* 103(4):1391-1397.

87. Novitskiy SV, *et al.* (2008) Adenosine receptors in regulation of dendritic cell differentiation and function. *Blood* 112(5):1822-1831.

88. Parmely MJ, *et al.* (1993) Adenosine and a related carbocyclic nucleoside analogue selectively inhibit tumor necrosis factor-alpha production and protect mice against endotoxin challenge. *J Immunol* 151(1):389-396.

89. Ezeamuzie CI & Khan I (2007) The role of adenosine A(2) receptors in the regulation of TNF-alpha production and PGE(2) release in mouse peritoneal macrophages. *Int Immunopharmacol* 7(4):483-490.

90. Hasko G, *et al.* (2000) Adenosine inhibits IL-12 and TNF-[alpha] production via adenosine A2a receptor-dependent and independent mechanisms. *FASEB J* 14(13):2065-2074.

91. Bshesh K, *et al.* (2002) The A2A receptor mediates an endogenous regulatory pathway of cytokine expression in THP-1 cells. *J Leukoc Biol* 72(5):1027-1036.

92. Hasko G, *et al.* (1996) Adenosine receptor agonists differentially regulate IL-10, TNF-alpha, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice. *J Immunol* 157(10):4634-4640.

93. Le Moine O, *et al.* (1996) Adenosine enhances IL-10 secretion by human monocytes. *J Immunol* 156(11):4408-4414.

94. Koscso B, *et al.* (2012) Adenosine augments IL-10 production by microglial cells through an A2B adenosine receptor-mediated process. *J Immunol* 188(1):445-453.

95. Lee JY, *et al.* (2006) Activation of adenosine A3 receptor suppresses lipopolysaccharide-induced TNF-alpha production through inhibition of PI 3-kinase/Akt and NF-kappaB activation in murine BV2 microglial cells. *Neurosci Lett* 396(1):1-6.

96. Romio M, *et al.* (2011) Extracellular purine metabolism and signaling of CD73-derived adenosine in murine Treg and Teff cells. *Am J Physiol Cell Physiol* 301(2):C530-539.

CHAPTER 2

CD73-GENERATED ADENOSINE FACILITATES *TOXOPLASMA GONDII* DIFFERENTIATION TO LONG-LIVED TISSUE CYSTS IN THE CENTRAL NERVOUS SYSTEM[2]

[2] Originally published in the *Proceedings of the National Academy of Sciences*.
Mahamed D. et al. 2012. PNAS. 109(40): 16312-17. Copyright © [2012] the National Academy of Sciences

ABSTRACT

Toxoplasma gondii is an obligate intracellular protozoan pathogen that traffics to the central nervous system (CNS) following invasion of its host. In the CNS, *T. gondii* undergoes transformation from a rapidly dividing tachyzoite to a long-lived, slow dividing bradyzoites-contained within cysts. The role of extracellular adenosine in *T. gondii* pathogenesis has not been previously investigated. *T. gondii* utilizes host purines such as adenosine for its energy needs, as it is unable to make its own. Here, we show that $CD73^{-/-}$ mice, which lack the ability to generate extracellular adenosine, are protected from *T. gondii* chronic infection, with significantly fewer cysts and reduced susceptibility to reactivation of infection in the CNS independent of host effector function. Parasite dissemination to the brain was unimpaired in $CD73^{-/-}$ hosts, suggesting that the reduced cyst number is due to impaired parasite differentiation in the CNS. Confirming this, *T. gondii* tachyzoites formed fewer cysts following alkaline pH stress in astrocytes isolated from $CD73^{-/-}$ mice compared to wildtype, and in fibroblasts treated with a CD73 inhibitor. Cyst formation was rescued in $CD73^{-/-}$ astrocytes supplemented with adenosine, but not with adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine. Further, mice lacking adenosine receptors had no defect in cyst formation. Based on these findings we conclude that CD73 expression promotes *T. gondii* bradyzoite differentiation and cyst formation by a mechanism dependent on the generation of adenosine, but independent of adenosine receptor signaling. Overall, these findings suggest that modulators of extracellular adenosine may be used to develop therapies aimed at defending against human toxoplasmosis.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan pathogen that traffics to the central nervous system (CNS) following initial invasion and replication in the gut (1). Infection with *T. gondii* commonly occurs in humans by ingestion of contaminated meat. In healthy individuals, the parasite forms tissue cysts, which limits its replication but enables the parasite to avoid immune cell-mediated destruction. Reactivation of latent infection in immunocompromised individuals and vertical transmission during pregnancy can lead to severe disease (2, 3). Dissemination of the parasite throughout the host is thought to be mediated by infected immune cells, which transport live parasites to the CNS and skeletal muscle where *T. gondii* establishes a chronic infection by differentiating into long-lived tissue cysts (4, 5).

Host cell-mediated immunity is the major deterrent against toxoplasmosis (6). The immune response in healthy individuals keeps *T. gondii* in check so that cyst-containing bradyzoites remain dormant in the CNS for the life of the host without overt clinical symptoms. This delicate balance between host and parasite survival is mediated both by host immune modulators and by *T. gondii* modification of host factors to promote its survival and transmission and to avoid excessive tissue damage leading to the host's demise (6-8).

Extracellular adenosine is a purine nucleoside generated by the sequential dephosphorylation of adenosine triphosphate (ATP) by the ectoenzymes CD39 and CD73 (reviewed in (9)). CD73 is a GPI-anchored cell-surface glycoprotein that catalyzes the final and rate-limiting conversion of adenosine monophosphate (AMP) to adenosine (10). Adenosine mediates its effects by binding to four 7-transmembrane receptors: A_1, A_{2A}, A_{2B} and A_3. Adenosine receptors and CD73 are highly expressed on various cell types, including immune cells and CNS resident cells (11). Extracellular adenosine signaling functions to prevent

excessive inflammation by suppressing pro-inflammatory cytokines, inhibiting leukocyte entry into tissues through downregulation of adhesion molecules and chemokines, and triggering the production of anti-inflammatory cytokines such as IL-10 (12-14). Further, CD73 expression and downstream adenosine signaling are critical for compensatory responses to tissue ischemia (13, 15, 16). Therefore, extracellular adenosine produced as a result of CD73 acts on adenosine receptors to regulate inflammation and protect against collateral tissue damage. Recent studies from our lab showed that CD73 and adenosine receptor expression on choroid plexus epithelial cells mediates T cell infiltration in the CNS, while expression on brain endothelial cells regulates blood-brain barrier function (17, 18).

The role of CD73 in *T. gondii* infection has not been previously explored. However, work by Blader *et al*. (19) showed that infection of human fibroblast with *T. gondii* two hours post infection resulted in the upregulation of genes associated with the immune response, including CD73. *T. gondii*, like other apicomplexa, need host purines, such as adenosine as they cannot synthesize their own (20). Two purine salvage pathways have been identified in *T. gondii*, involving the enzymes hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) and adenosine kinase (AK)(21). *T. gondii* adenosine kinase (AK) activity is 10-fold higher than other purine salvage enzymes; and adenosine is the preferred source of purines for *T. gondii (22)*. This suggests that host derived adenosine plays an important role in *T. gondii* pathogenesis**.** In this study, we set out to determine if CD73 is thus important for *T. gondii* pathogenesis. Interestingly, we found that CD73-knockout mice are less susceptible to chronic *T. gondii* infection, exhibiting reduced morbidity and mortality and markedly reduced cyst burden in the brain, compared to WT control mice. In an *in vitro* cell culture model that recapitulated the *in vivo* model, we found that addition of adenosine, but not activation of adenosine receptors

rescued cyst formation. Our findings suggest that CD73 contributes to *T. gondii* persistence in the CNS by promoting parasite differentiation.

MATERIALS AND METHODS

Mouse strains and *in vivo* infection

Female C57BL/6 WT and CD73-knockout mice (11), provided by Dr. Linda Thompson (Oklahoma Medical Research Foundation, Oklahoma City), were bred in specific pathogen free conditions at Cornell University. Adenosine receptor A1 and A2A knockout mice were a gift from Dr. Jurgen Schnermann (NIH/NIDDK, Bethesda, MD) and Dr. Jiang-Fan Chen (Boston University School of Medicine, Boston, MA), respectively. All animal experiments were approved by Cornell University's Institutional Animal Care and Use Committee For chronic *T. gondii* infection, mice were inoculated per-orally with 10 or 20 cysts of the ME49 strain and monitored for weight loss, survival and parasite burden. For determination of parasite cyst burden, *T. gondii* infected mice were euthanized and their brains were harvested and homogenized. Brain homogenates were centrifuged at 250 × g for 10 minutes, resuspended in PBS and aliquots were mixed with an equal volume of Lugol's iodine for counting cysts using light microscopy.

Parasite strains and maintenance

Cysts of the type II *T. gondii* ME49 strain were maintained in outbred Swiss Webster mice (Taconic) (53). Tachyzoites of the type II ME49 and PTG strains were passaged in human foreskin fibroblasts (HFFs)(53) in DMEM media supplemented with 3% v/v fetal calf serum, 1 mM penicillin/streptomycin, 1 mM L-glutamine, 25 mM HEPES buffer and 2-mercaptoethanol. Bradyzoites of the type II ME49 strain were isolated from cysts of infected Swiss Webster mice

through pepsin-HCl digestion (54), followed by passage in HFF fibroblasts 3-4 times before use in experiments. Parasites were purified from freshly lysed HFF monolayers by filtration with a syringe-top 3 μm pore filter and centrifugation at 500 × g for 10 minutes at 4°C. Parasite pellets were resuspended in complete DMEM with 3% FCS and counted using a haemocytometer.

Histology

Brains from infected mice were fixed in 10% formalin and processed detection of T. gondii cysts at the Animal Health Diagnostic Center in the College of Veterinary Medicine's Histology Core Facility of Cornell University. Polyclonal rabbit anti-T. gondii antibody was used to visualize cysts and the sections were counterstained with hematoxylin.

Flow cytometry

Immune cells isolated from the brain (as described above) were stained with fluorochrome-conjugated antibodies against CD3 (clone 17A2), CD11b (clone M1/70), and CD45 (clone 30-F11). Astrocytes isolated from the brain (as described above) and infected or uninfected with *T. gondii* were stained with fluorochrome-conjugated antibodies against SAG-1 (mouse monoclonal anti-p30) and CD73 (rat TY/11.8). Human foreskin fibroblasts were stained with phycoerythrin-conjugated anti-human CD73 (mouse AD2). Antibodies were purchased from eBioscience (CD3, CD11b, mouse CD73), BD Bioscience (CD45, human CD73), and Argene (SAG1). Data was acquired on a FACS Canto II, and analyzed with FlowJo software.

Quantitation of Gene Expression by qPCR

Cultured astrocytes and tissues from mice were homogenized in TRIZOL Reagent (Invitrogen Life Technologies) and stored at -80°C prior to total RNA extractions according to the manufacturer's protocol. RNA was treated with DNase (Epicentre Biotechnologies) before

determination of nucleic acid concentration and cDNA synthesis with the High Capacity Reverse Transcription Kit (Applied Biosystems). Brain or astrocyte-derived cDNA was analyzed for host and parasite genes using real-time PCR SYBR Green Master Mix from KAPA Biosystems using appropriate primers (Table S1 (55)) and run on a BioRad CFX96 real time qPCR system. Gene expression was normalized to the housekeeping gene PGK1 and calculated using the $2^{-\Delta Ct}$ method. Melt curve analyses were performed to ensure the specificity of qPCR product.

Quantitation of *T. gondii* by qPCR

Harvested brain and small intestine tissue samples from *T. gondii* infected mice were homogenized in PBS, pelleted and then digested (50-100 mg tissue samples) with Proteinase K (0.2 mg/ml) in lysis buffer (10 mM Tris pH 8.0, 100 mM NaCl, 25 mM EDTA, 0.5% SDS) overnight at 55°C. Genomic DNA was precipitated with isopropanol, washed with 75% ethanol, and resuspended in molecular grade water. Nucleic acid concentration was determined and quantitative PCR (qPCR) was performed using 100 ng of gDNA in KAPA real-time SYBR Green mastermix (Kapa Biosystems) and 200 nM primers for the multi-copy B1 gene of *T. gondii* (Table S1).A standard curve was generated using known numbers of tachyzoites, extracted as described above.

Bradyzoite isolation

Wildtype and $CD73^{-/-}$ mice were orally infected with 10 ME49 cysts. Eight weeks after infection, brains were homogenized in PBS and cysts isolated as described in Materials and Methods. Bradyzoites were liberated using pepsin-HCl.

***T. gondii* proliferation**

To assess *T. gondii* proliferation (40), isolated astrocytes (as described above) and human foreskin fibroblasts (Hs27) were seeded into 96-well tissue culture plates and allowed to grow to confluence before inoculation with tachyzoites (PTG or ME49) or freshly isolated bradyzoites (ME49) at various multiplicities of infection (MOI) in complete DMEM with 3% FCS. Seventy two post-infection, 1 μCi of [5,6-^{3}H] uracil was added to each well and plates were incubated for an additional 24 h. Plates were centrifuged at 500 × g, supernatants removed, and the cells were lysed with 0.5 M NaOH. Nucleic acids were precipitated with ice-cold 5% trichloroacetic acid before transfer to glass fiber filters with a Tomtec Mach II M cell harvester. Incorporation of radioactive uracil was determined utilizing a liquid scintillation beta counter (Perkin-Elmer Life Sciences).

Astrocyte isolation and culture

To generate astrocytes *in vitro*, one to three day old wild type and CD73$^{-/-}$ mice were euthanized and the brains were removed aseptically. Brains were minced, triturated in PBS and homogenized with passage through an 18g blunt needle. Astrocytes were enriched through Percoll gradient centrifugation. Brain homogenates were resuspended in 30% Percoll and layered over 70% Percoll before centrifugation at 600 × g for 20 minutes at room temperature. The 30-70% interface was collected and washed in PBS. Cells were resuspended in complete DMEM (with 1.5 g/L sodium bicarbonate, 10% fetal calf serum v/v, 1 mM penicillin and streptomycin, 1 mM L-glutamine, 25 mM HEPES buffer and 2-mercaptoethanol) and transferred to tissue culture-treated flasks. After 24 hours of incubation at 37°C to allow for astrocyte attachment, media was replaced with fresh complete DMEM and cells were allowed to grow to 80% confluence with periodic feeding with the same media. Microglial cells were detached and removed by 200 rpm shaking at 37°C overnight and fresh media replacement. Astrocytes were

collected by trypsinisation and seeded onto sterile glass cover-slips for immunofluorescent staining experiments, 48-well plates for gene expression experiments, and 96-well plates for parasite proliferation assays.

Dexamethasone Treatment

Wildtype and CD73$^{-/-}$ mice were infected with 10 cysts of *T. gondii* ME49. Thirty days post-infection, mice were given 5 mg/L dexamethasone 21-phosphate disodium salt (Sigma-Aldrich, St. Louis, MO, USA) in drinking water. Water was replaced every second day, and mice were weighed and monitored for morbidity and mortality.

***T. gondii* differentiation and immunofluorescence microscopy**

To assess *T. gondii* differentiation *in vitro* (45), astrocytes isolated from WT and CD73$^{-/-}$ neonates (as described in (56) and detailed in Text S1) were grown to confluence on sterile coverslips in 24-well tissue culture plates. The cells were then infected at a MOI of 1:2 with ME49 tachyzoites for 2 days at pH 7.2 in complete DMEM with 3% FCS at 37°C and 5% CO_2. The media was then replaced with complete DMEM-3% FCS at pH 8.0 to induce bradyzoite differentiation (replaced every 3 days to maintain alkaline conditions), and the cells were cultured at 37°C in air. In some cases the media was supplemented with adenosine (50 µM), 5′-N-Ethylcarboxamidoadenosine (NECA, 10 µM), alpha,beta-methylene ADP (meADP, 50 µM) or vehicle (DMSO) during the *T. gondii* differentiation before fixing and staining the cells at 12 days post-alkaline pH challenge. For the differentiation time course, the cells were analyzed at 2, 4, and 8 days post-pH increase. Cells were fixed in 2% paraformaldehyde for 20 minutes at room temperature, then blocked and permeabilized with 10% normal rabbit serum and 0.2% Triton-X 100 in PBS with 1% BSA. Intracellular staining was performed overnight at 4°C with

rabbit anti-BAG1 (J.P. Dubey, USDA, Beltsville, Maryland) followed by goat anti-rabbit conjugated to Alexa Fluor 488 or Texas Red X (Invitrogen) and either *Dolichos biflorus* agglutinin (DBA) conjugated to rhodamine (Vector Labs, Burlingame, California) or mouse anti-SAG1-FITC. Coverslips were washed and mounted with Vectashield Mounting Medium with DAPI (Vector Labs). Images were obtained on a Zeiss Axio Imager M1 fluorescent microscope utilizing AxioVision software. For quantitation of cyst numbers, 25 adjacent fields per slide were scanned and the number of BAG1+DBA+ cysts enumerated.

Data analysis

Statistical analyses were performed with Graphpad Prism software. Two-tailed Student's T-tests or 2-way ANOVA with Bonferroni post-tests were used to compare differences between groups. Kaplan Meier survival curves were compared with the Mantel-Cox log rank test. Differences with a p-value below 0.05 were considered significant.

RESULTS

CD73$^{-/-}$ mice are less susceptible to toxoplasmosis

To determine the role of CD73 in *T. gondii* infection, C57BL/6 (WT) and CD73$^{-/-}$ mice (11) were infected with 10 cysts of *T. gondii* ME49 strain (23) by oral gavage, and then monitored for survival, weight loss, and cyst burden. In WT mice, infection with this dose of parasites allows survival through acute infection (<14 days) and establishment of chronic infection. However, WT mice are susceptible to parasite reactivation in the CNS, and the animals ultimately succumb to toxoplasmic encephalitis. CD73$^{-/-}$ mice were significantly more resistant to chronic *T. gondii* infection than WT mice (Figure 2.1A-D). While both WT and CD73$^{-/-}$ mice infected with a low dose of the ME49 strain of *T. gondii* survived the acute infection, WT but not

CD73$^{-/-}$ mice died during chronic infection, with the onset of morbidity and mortality occurring 8-10 weeks post-infection (Figure 2.1A). Weight loss monitored during infection confirmed that WT mice exhibited increased susceptibility to toxoplasmosis, losing significantly more weight during both acute and chronic infection (Figure 2.1B). In addition, the increased survival observed in CD73$^{-/-}$ mice correlated with decreased parasite burden in the CNS during chronic infection (Figure 2.1C-D). Gene expression analysis showed that CD73 is expressed in the brain and that its expression increased over the course of infection in WT mice (Figure 2.1E). Therefore, these results suggest that CD73 promotes parasite persistence in the brain resulting in increased susceptibility to toxoplasmic encephalitis.

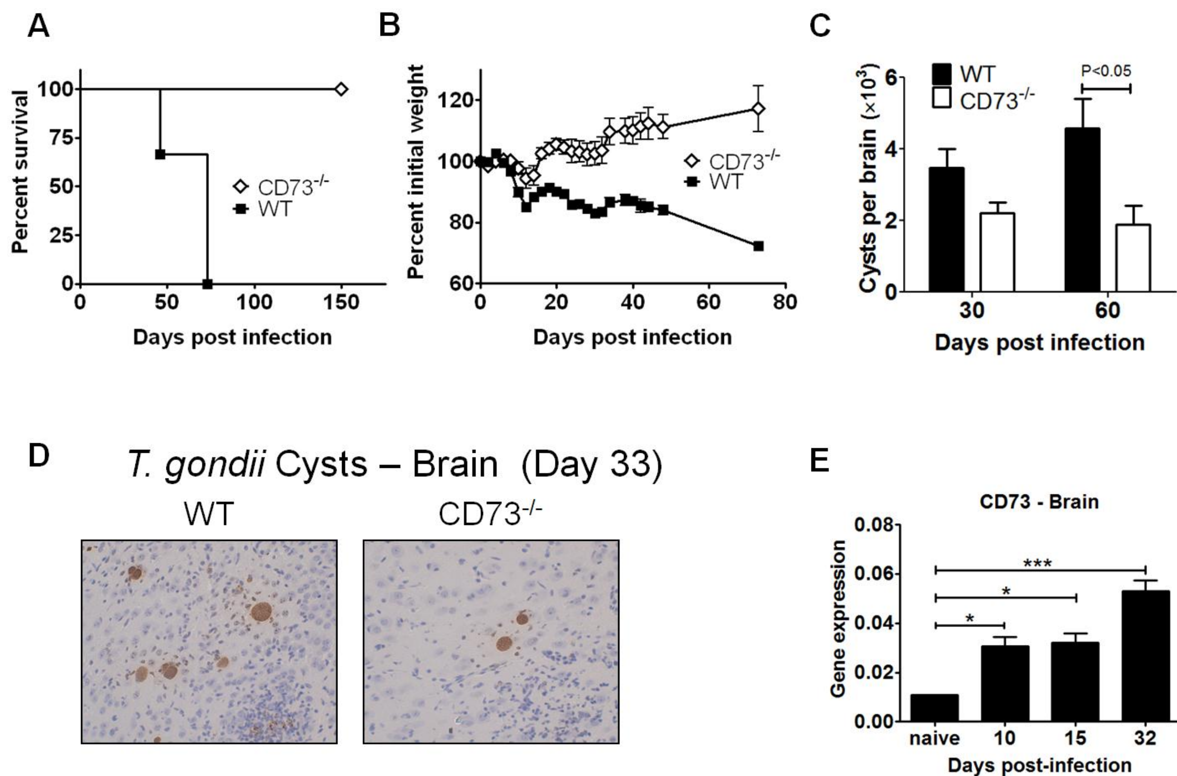


Figure 2.1 CD73$^{-/-}$ mice are resistant to chronic *T. gondii* infection. WT and CD73$^{-/-}$ mice were infected with 10 cysts of the *T. gondii* ME49 strain and then monitored for (A) survival and (B) weight loss. (C) Cyst burden in infected mouse brains was quantified by light microscopy.

(D) Brain sections from *T. gondii* infected mice were stained by immunohistochemistry for *T. gondii* cysts and are shown with representative cyst distribution near the hippocampus from Day 33 post infection (*T. gondii* cysts = brown, hematoxylin-stained nuclei = blue). (E) CD73 gene expression in WT mice during infection. Expression was normalized to the PGK1 housekeeping gene. Error bars represent the s.e.m. Significant differences based on two-tailed t-tests are displayed (* $P<0.05$, ** $P<0.01$, *** $P<0.005$). The data shown is representative of 3 experiments using 3-5 mice per group per time point.

CD73$^{-/-}$ and WT mice show similar levels of *T. gondii* parasite burden in the brain during the acute but not chronic stages of infection.

To assess whether the reduced parasite burden in CD73$^{-/-}$ mice could be explained by failure of the parasite to establish infection in the intestinal ileum or to disseminate to the brain following oral infection, WT and CD73$^{-/-}$ mice were infected with 20 ME49 cysts, then sacrificed at various time-points post infection to determine parasite burden in the ileum of the small intestine, liver and brain by quantitative real-time PCR. Both strains of mice showed similar kinetics of parasite expansion and clearance during the acute stage of infection in the gut, as well as similar dissemination to the brain (Figure 2.2A and 2.2B). In the ileum of WT and CD73$^{-/-}$ mice, *T. gondii* DNA was consistently detectable by day 3, peaked on day 7, then decreased significantly by day 14 (Figure 2.2A). Liver parasite burden similarly peaked at 7 days in both strains of mice before decreasing significantly by day 14 (Figure 2.2B) Parasite dissemination to the brain was detectable by day 7 post-infection, and then increased markedly thereafter (Figure 2.2C). Despite the similar initial kinetics of *T. gondii* dissemination to the brain, PCR results confirmed that CD73$^{-/-}$ mice had significantly reduced parasite burden in the brain during chronic disease compared to WT mice (Figure 2.2D). This reduced parasite burden in CD73$^{-/-}$ mice was consistent with their reduced susceptibility to latent infection (Figure 2.1).

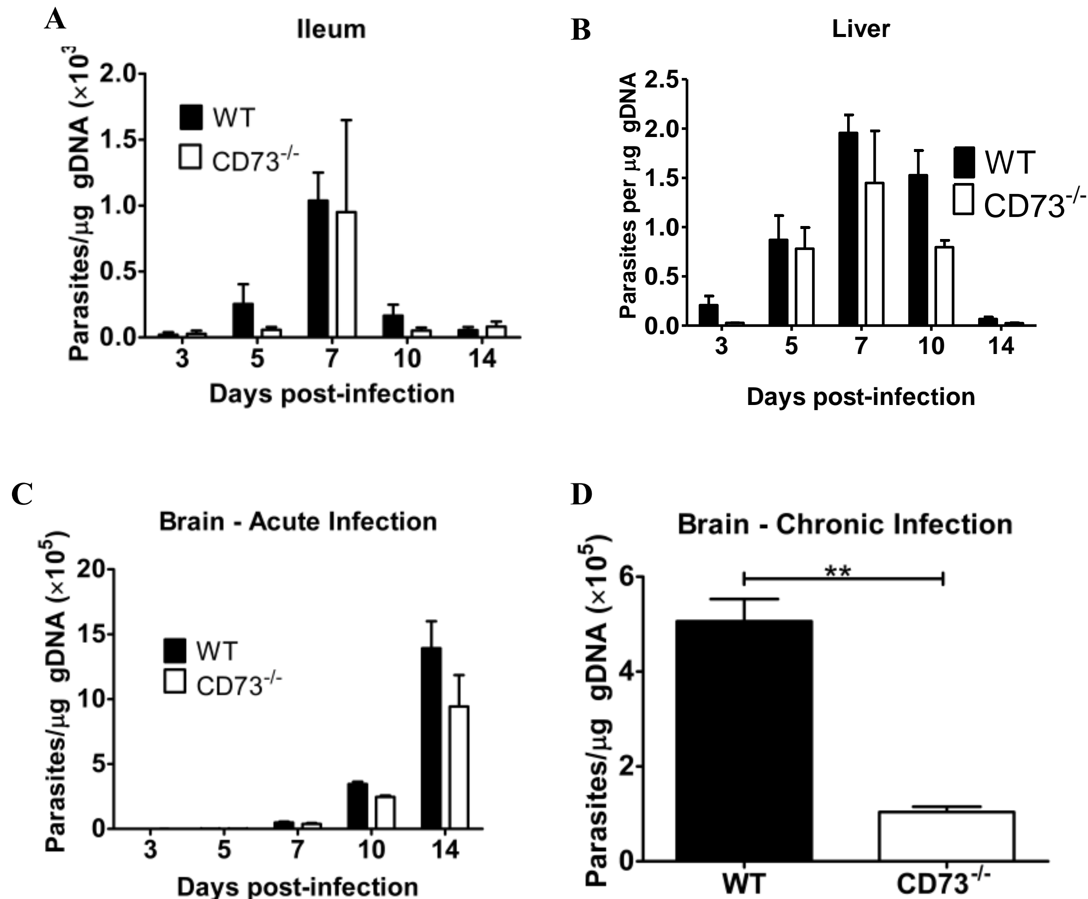


Figure 2.2 CD73$^{-/-}$ mice have reduced parasite burden in the brain during chronic *T. gondii* infection. WT and CD73$^{-/-}$ mice were infected with 20 cysts of the *T. gondii* ME49 strain and then sacrificed at various time-points during (A-C) acute and (D) chronic infection to determine parasite burden in the (A) small intestine ileum (A), liver (B) and brain (C and D) by real-time quantitative PCR. Error bars represent the s.e.m. Significant differences based on two-tailed t-tests are displayed (** $P<0.01$). The data shown represents one experiment with 3-5 mice per group per time point.

$CD73^{-/-}$ and WT mice have a similar degree of leukocyte infiltration into the brain during *T. gondii* chronic infection.

As previously reported (24), infection with *T. gondii* in WT mice is associated with infiltration of leukocytes into the brain by day 10 post-infection and remains elevated throughout the chronic stage of disease (Figure 2.3 *top*). The absolute number of leukocyte recruitment into the brains of WT and $CD73^{-/-}$ mice was similar (Figure 2.3 *top left*), although a decrease in T cells (as a percentage of total $CD45^{+}$ cells) was observed in $CD73^{-/-}$ mice early in infection at day 10 (Figure 2.3 *top center*), with a relative increase in $CD11b^{+}$ cells (Figure 2.3 *top right*). Since overall leukocyte numbers and composition was similar in $CD73^{-/-}$ compared to WT mice during chronic infection, the reduction in parasite burden observed in $CD73^{-/-}$ mice at the same time points (Figure 2.1) was likely not due to increased leukocyte recruitment to the CNS.

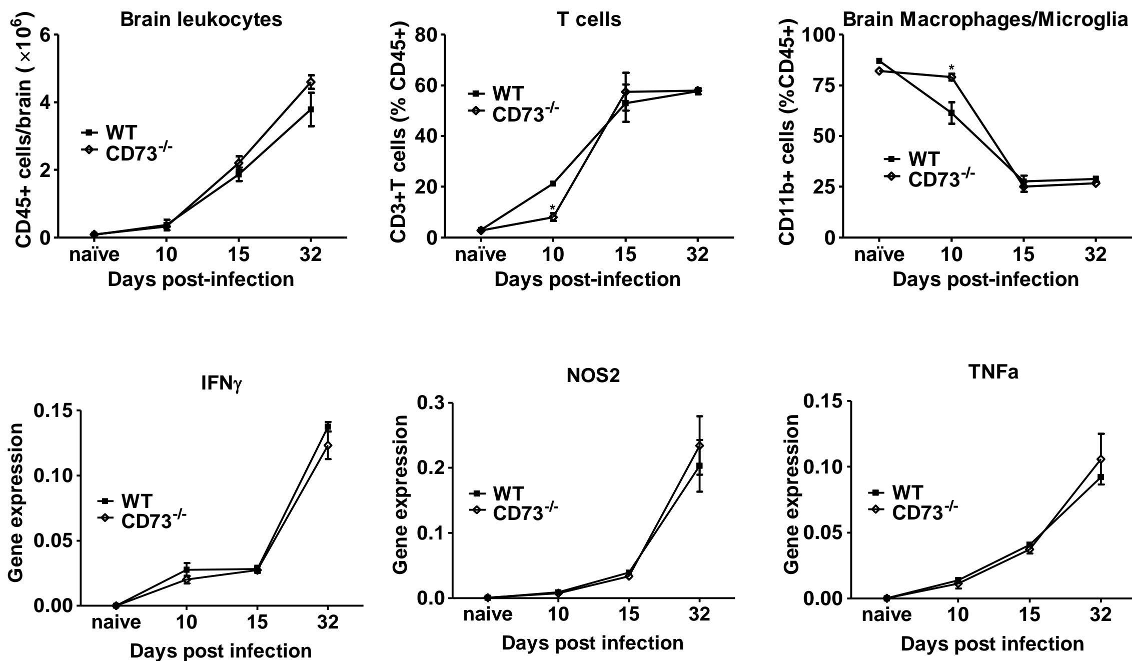


Figure 2.3. **Kinetics of the immune response to *T. gondii* infection in the brain**. Wild type and $CD73^{-/-}$ mice were infected with 10 cysts of the *T. gondii* ME49 strain and then sacrificed at

various time-points to determine brain leukocyte infiltration (top panels) and effector gene expression (bottom panels). Gene expression levels were normalized to the PGK1 housekeeping gene. Error bars represent the s.e.m. The data shown is representative of two experiments using 2-5 mice per time point.

Brains from *T. gondii* infected CD73$^{-/-}$ and WT mice have similar immune effector gene expression kinetics.

We next analyzed gene expression of effector molecules associated with *T. gondii* infection to determine if the resistance of CD73$^{-/-}$ mice was mediated by an increased production of these molecules. Interferon gamma (IFNγ) is a cytokine critical for controlling *T. gondii* expansion and reactivation in the brain (25-29). In WT and CD73$^{-/-}$ mice, we observed an equally substantial and sustained increase in IFNγ mRNA expression during the course of *T. gondii* infection (Figure2.3, *bottom left*). Another effector molecule important in the killing and inhibition of *T. gondii* growth is inducible nitric oxide synthase (iNOS) found in peripheral macrophages and the CNS (30). *T. gondii* infection led to increased expression of the iNOS gene, NOS2, in WT and CD73$^{-/-}$ mice to a similar extent (Figure 2.3, *bottom center*). Likewise, no difference was observed during *T. gondii* infection in the upregulation of TNF-α mRNA, which is important in the control of toxoplasmosis in synergy with IFNγ (31) between WT and CD73$^{-/-}$ mice (Figure 23, *bottom right*). These findings suggest that gene expression of effector molecules in the brain are not altered in the absence of CD73 and therefore are not responsible for protecting CD73$^{-/-}$ mice from *T. gondii* chronic infection.

Expression of key adhesion molecules and chemokines in the CNS during *T. gondii* infection.

Leukocyte infiltration into the CNS is mediated by chemokines and adhesion molecules secreted by brain endothelial cells, astrocytes and microglial cells in response to infection and autoimmune inflammation (32). Thus we evaluated expression of three key factors that have been identified in mediating immune cell entry into the CNS in response to infection and neuroinflammatory diseases. While MadCAM1 expression in the CNS was markedly upregulated by *T. gondii* infection, consistent with its role in promoting leukocyte adhesion to brain endothelium (33-35), no significant difference in the increases in MadCAM1 gene expression was observed between infected wild type and CD73$^{-/-}$ mice (Figure 2.4 *left*). Vascular adhesion molecule 1 (VCAM1), which has been implicated in recruitment of immune cells during *T. gondii* infection (36, 37), also increased over the course of *T. gondii* infection in both wild type and CD73$^{-/-}$ mice (Figure 2.4 *middle*). Interestingly, VCAM1 expression was reduced early (Day 10 and 15) during *T. gondii* infection in the brains of CD73$^{-/-}$ mice (Figure 2.4 *middle*), which correlates with the reduced T cell infiltrates observed in the brains of CD73$^{-/-}$ mice early in infection (Figure 2.3 *top middle*). Finally, the adhesion molecule/chemokine CX3CL1 (fractalkine), which is highly expressed in the CNS and recruits brain resident microglia and leukocytes into the CNS (32, 38, 39), was observed in the brains of naïve and infected mice, although CX3CL1 gene expression in wild type and CD73$^{-/-}$ mice decreased at a similar level over the course of *T. gondii* infection (Figure 2.4 *right*). These findings suggest that the changes in gene expression of adhesion molecules in the brain are not dependent on CD73 and therefore are not responsible for protecting CD73$^{-/-}$ mice from *T. gondii* chronic infection.

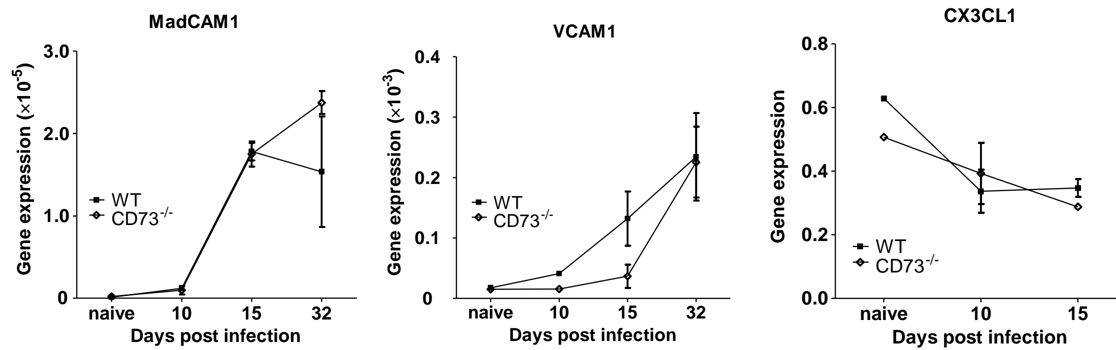


Figure 2.4. **Kinetics of MadCAM1, VCAM1 and CX3CL1 (fractalkine) expression in the brain following *T. gondii* infection**. Wild type and CD73$^{-/-}$ mice were infected with 10 cysts of the *T. gondii* ME49 strain and then sacrificed at various time-points to determine brain MadCAM1, VCAM1, and CX3CL1 gene expression levels. Gene expression levels were normalized to the PGK1 housekeeping gene. Error bars represent the s.e.m. The data shown is representative of two experiments using 2-5 mice per time point.

Lack of CD73 enhances proinflammatory cytokine responses to *T. gondii*.

To assess whether the decreased disease susceptibility of CD73$^{-/-}$ mice to chronic *T. gondii* infection was associated with modified secretion of key proinflammatory cytokines which control toxoplasmosis, we cultured lymphocytes from the spleen, lymph nodes, and brain of wild type and CD73$^{-/-}$ mice infected with *T. gondii* and restimulated the cells with soluble tachyzoite derived antigens (STAg). Lymphocytes isolated from the spleen, lymph nodes, and brains of CD73$^{-/-}$ infected mice produced significantly higher levels of interleukin 12 (IL-12) upon restimulation compared to those from infected wild type mice (Figure 2.5 *left*). Consistent with increased IL-12 levels, IFNγ production was also increased in restimulated isolated lymphocytes from CD73$^{-/-}$ mice compared to wild type (Figure 2.5 *right*). These data suggest that

lymphocytes from *T. gondii* infected CD73$^{-/-}$ mice can produce more proinflammatory cytokines compared to those from wild type mice.

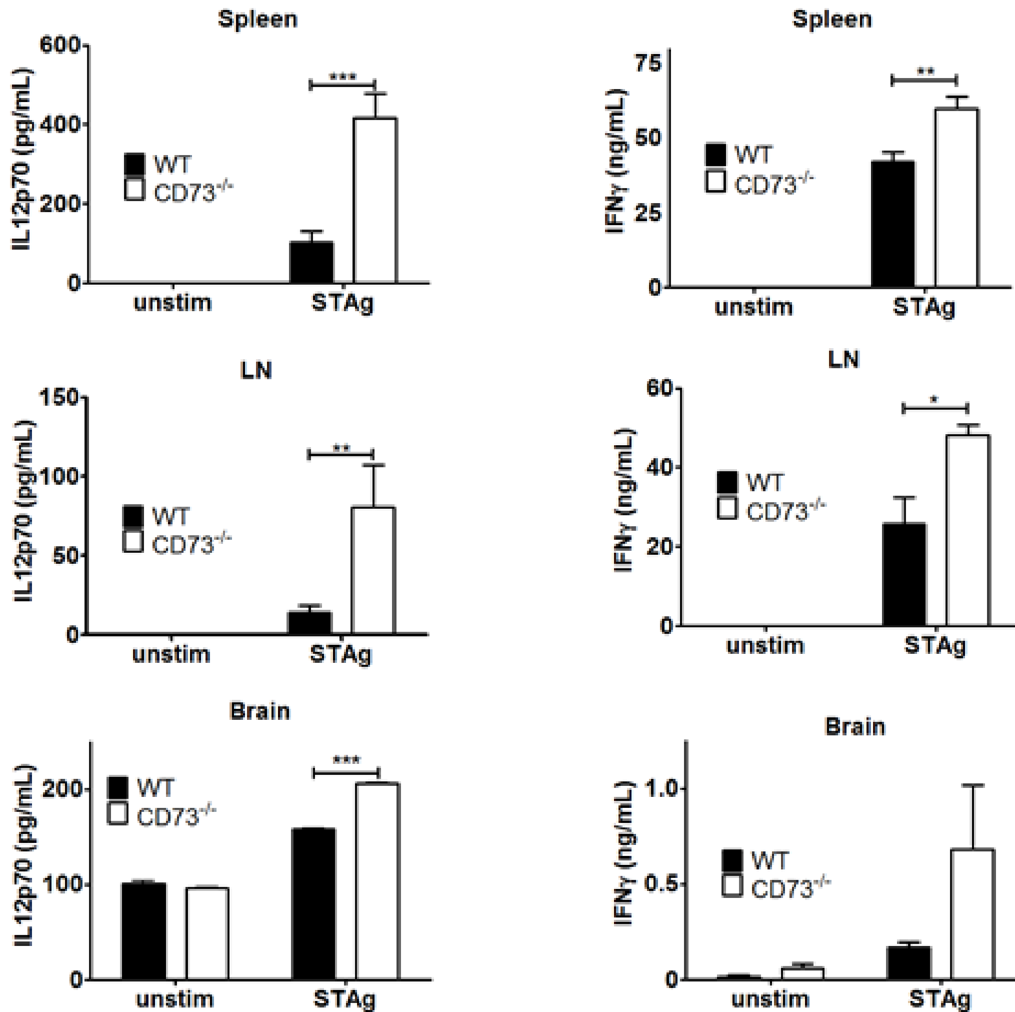


Figure 2.5. Proinflammatory cytokine production from isolated and restimulated lymphocytes following chronic infection with *T. gondii*. Wild type and CD73$^{-/-}$ mice were infected with 10 cysts of the *T. gondii* ME49 strain and then sacrificed during the chronic stage of infection (day 32). Spleen, lymph node (LN) and brain leukocytes were isolated and restimulated in vitro with 20 µg/ml of soluble tachyzoite antigens (STAg) or media alone (unstim) and the generation of IL-12 and IFNγ was measured by ELISA. Error bars represent the

s.e.m. Significant differences based on two-tailed t-tests are displayed (* P<0.05, ** P<0.01, *** P<0.005). The data shown represents one of two experiments using 2-7 mice per group.

Infection and proliferation of *T. gondii* in astrocytes is not dependent on CD73 expression

To assess whether CD73$^{-/-}$ cells in the CNS were less permissive for *T. gondii* infection or proliferation, we isolated WT and CD73$^{-/-}$ astrocytes and assessed the ability of the parasite to infect and proliferate in these cells. Astrocytes from WT and CD73$^{-/-}$ neonates were cultured and expanded *in vitro* and then infected with tachyzoites of the type II *T. gondii* strain PTG (Figure 2.6A). Flow cytometric analysis of astrocytes from WT mice revealed two distinct populations of cells based on CD73 expression, with approximately 40% of the cells being CD73-positive (Figure 2.6A). Likewise, the proliferation of tachyzoites of the PTG and ME49 strains of *T. gondii*, as measured by ^{3}H-uracil incorporation (40), was similar in both WT and CD73$^{-/-}$ infected astrocytes (Figure 2.6B and C). These results indicate that *T. gondii* infection and replication of tachyzoites in astrocytes is not dependent on CD73 expression.

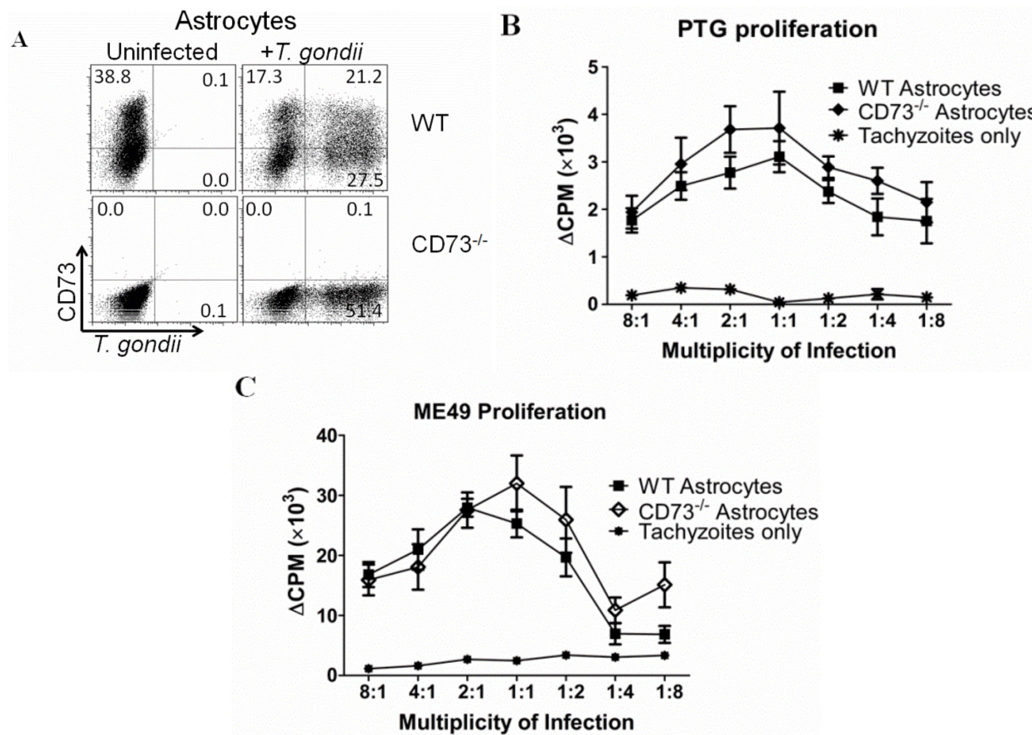


Figure 2.6. Proliferation of T. gondii in cultured astrocytes from wild type and CD73$^{-/-}$ mice. Astrocytes from wild type and CD73$^{-/-}$ neonates were cultured and infected with *T. gondii* PTG strain tachyzoites. (A) Comparison of CD73 expression and T. gondii infection level (SAG1) in cultured astrocytes 72 h after infection. (B) and (C) Proliferation of tachyzoites of the PTG (B) and ME49 (C) strains of *T. gondii* in cultured astrocytes at varying multiplicities of infection (MOI) as measured using the uracil incorporation assay. Incorporation into uninfected astrocytes was subtracted from the counts per minute to generate ΔCPM. Error bars represent the s.e.m. The data shown represents one of three experiments.

CD73 expression promotes bradyzoite/cyst persistence in the brain.

Since *T. gondii* infected CD73$^{-/-}$ mice have a reduced number of brain cysts compared to WTs (Figure 2.1), we next determined whether CD73 expression is required for *T. gondii* bradyzoite maturation. We isolated brains of *T. gondii* infected WT and CD73$^{-/-}$ mice during

acute (day 10 and day 15) and chronic (day 30) infection and determined mRNA expression of the SAG1 (tachyzoite specific) and BAG1 (bradyzoite specific) genes by qPCR (41-43). In both WT and CD73$^{-/-}$ mice, tachyzoite SAG1 was highly expressed on day 10 but decreased on days 15 and 30 (Figure 2.7A). Conversely, bradyzoite BAG1 was expressed at low levels on day 10 and highly expressed on days 15 and 30 in both WT and CD73$^{-/-}$ mice (Figure 2.7B). Interestingly, while BAG1 expression was highly expressed in both mouse strains at day 30 post infection, mice that lacked CD73 had significantly lower brain BAG1 expression levels compared to WT mice (Figure 2.7B). These results suggest that bradyzoites are generated in the brains of CD73$^{-/-}$ mice similar to WT but do not persist or proliferate. To test the viability of encysted bradyzoites, we purified cysts from chronically infected WT and CD73$^{-/-}$ mice and liberated bradyzoites using enzymatic digestion. These bradyzoites were used to infect fibroblasts, and their proliferation assessed using ^{3}H uracil incorporation. We found that bradyzoites isolated from CD73$^{-/-}$ hosts proliferate significantly less than those isolated from WT hosts (Figure A1). However, *in vivo*, the few cysts found in CD73$^{-/-}$ hosts were capable of recrudescence if mice were treated with dexamethasone to induce immunosuppression (44) (Figure A2). Thus, while there is evidence that parasites isolated from CD73$^{-/-}$ mice proliferate less well *in vitro*, this is not enough to significantly impact the outcome of dexamethasone-induced toxoplasmic encephalitis.

CD73 promotes tachyzoite to bradyzoite differentiation in astrocytes

We next determined whether *T. gondii* differentiation from tachyzoites to bradyzoites might be impaired in resident CNS cells lacking CD73. Astrocytes from WT and CD73$^{-/-}$ mice were isolated, cultured and then infected with ME49 tachyzoites (Figure 2.7C and Figure 2.8). After 8 days in culture, we could detect robust expression of constitutive (Tubulin 1), tachyzoite-

specific (SAG1) and bradyzoite-specific genes (BAG1 and ENO1) in WT and CD73$^{-/-}$ cell cultures (Figure 2.7C). Interestingly, expression of the constitutive gene Tubulin 1 was slightly decreased in CD73$^{-/-}$ infected cells, while SAG1, BAG1 and ENO1 expression were significantly reduced in CD73$^{-/-}$ cells compared to WT cells. The reduction in bradyzoite genes (BAG1 and ENO1) in CD73$^{-/-}$ host cells was significantly pronounced at this time-point.

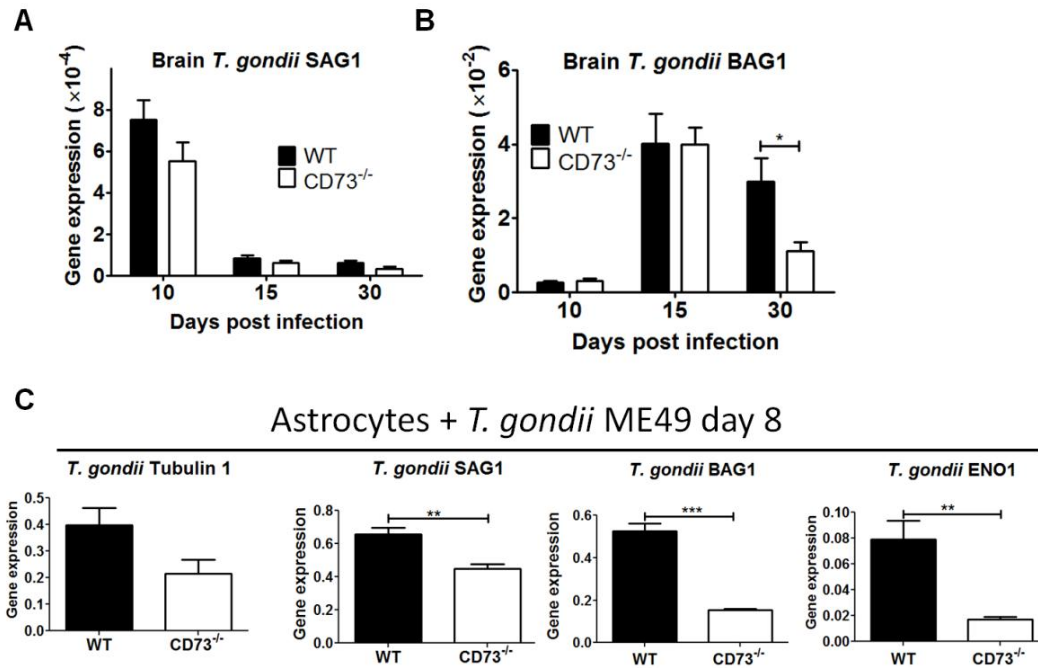


Figure 2.7. Gene expression profile of *T. gondii* tachyzoites and bradyzoites in the brains of infected WT and CD73$^{-/-}$ mice and in cultured brain astrocytes. (A and B) WT and CD73$^{-/-}$ mice were infected with 10 cysts of the *T. gondii* ME49 strain and then sacrificed at various time-points during acute (day 10 and 15) and chronic (day 30) infection to determine the brain expression of the (A) tachyzoite SAG1 and (B) bradyzoite BAG1 genes. The data shown represents one of three experiments using 2-5 mice per time point. (C) Astrocytes were cultured from neonatal WT and CD73$^{-/-}$ pups and then infected with ME49 tachyzoites to assess spontaneous differentiation of *T. gondii in vitro* by quantitative real-time PCR analysis of

constitutively expressed Tubulin 1, tachyzoite-specific SAG1 and bradyzoite-specific BAG1 and ENO1 genes. Parasite gene expression was normalized to host PGK1 housekeeping gene. Error bars represent the s.e.m. Significant differences based on two-tailed t-tests are displayed (* $P< 0.05$, ** $P<0.01$, *** $P<0.0001$).

To determine whether the differentiation of *T. gondii* in CD73$^{-/-}$ host cells was also impaired under bradyzoite-inducing conditions, we again cultured astrocytes from WT and CD73$^{-/-}$ hosts and infected them with *T. gondii* ME49 tachyzoites. At 2 days post infection, the pH of the culture media was increased from 7.2 to 8.0-8.1, to induce cyst formation (45). Confirming our quantitative gene expression data (Figure 2.7C) some tachyzoites (SAG1^{+}) spontaneously differentiated to bradyzoites (BAG1^{+}) even before alkaline pH challenge (Figure 2.8A left panel). Nevertheless, pH induction triggered substantial bradyzoite differentiation, with cyst formation observed after only 2 and 4 days in alkaline media (Figure 2.8A second and third panels), and mature cysts by 8 days post-induction (Figure 2.8A fourth panel and Figure 2.8C). Mature cysts, defined as positive for the bradyzoite marker BAG1 as well as the cyst wall component that binds *Dolichos biflorus* agglutinin (DBA) were quantified to compare the ability of astrocytes from WT and CD73$^{-/-}$ mice to support cyst formation *in vitro*. Significantly more cysts were observed in astrocytes cultures from WT mice compared to those from CD73$^{-/-}$ mice (Figure 2.8C). Consistent with this finding, analysis of BAG1 expression at the mRNA level by qPCR revealed significantly higher expression of this bradyzoite *T. gondii* gene in WT compared to CD73$^{-/-}$ astrocyte cultures (Figure 2.8B). These results strongly suggest that CD73 expression is required for the efficient formation or maintenance of cysts in astrocytes.

Exogenous adenosine rescues *T. gondii* cyst formation in CD73-deficient host cells

To investigate the mechanism by which CD73 promotes cyst formation or bradyzoite differentiation *in vitro*, we next determined whether exogenous adenosine, which mediates both adenosine receptor signaling as well as serving as a source of purines for parasites such as *T. gondii* can rescue the defect in bradyzoite maturation observed in mice and cells lacking CD73. We first determined by real-time PCR that three of the four adenosine receptors are expressed on astrocytes in addition to the ecto-enzymes CD39 and CD73 that generate adenosine from the breakdown of ATP (Figure 2.9A). As described above, astrocytes from WT and CD73$^{-/-}$ mice were isolated, cultured and then infected with ME49 tachyzoites in increased pH medium (from 7.2 to 8.0), to induce cyst formation in the presence of adenosine or vehicle control (DMSO). We observed that exogenous adenosine was able to significantly rescue *T. gondii* cyst formation in CD73$^{-/-}$ astrocytes to levels similar to WT but not vehicle control (Figure 2.9B).

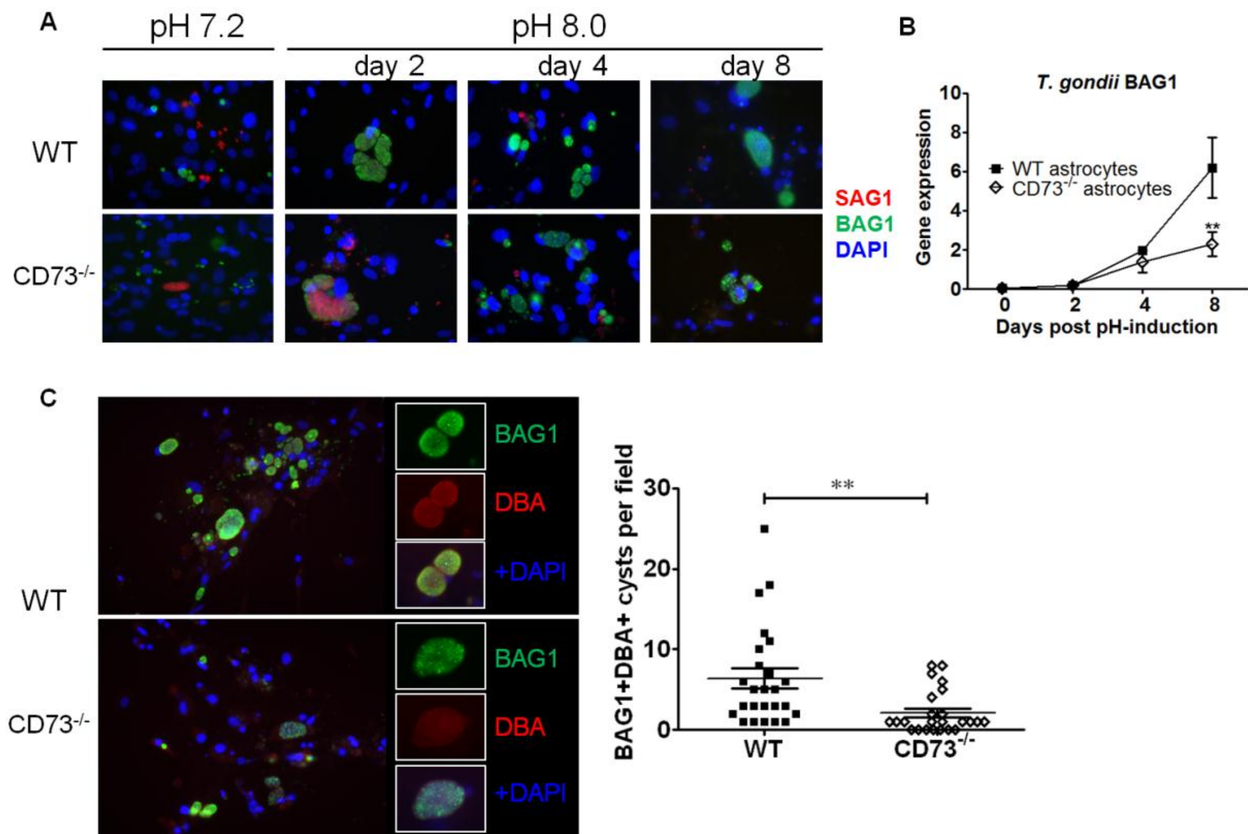


Figure 2.8. Differentiation of *T. gondii* in cultured astrocytes from WT and CD73$^{-/-}$ mice.

Astrocytes from WT and CD73$^{-/-}$ neonates were cultured and infected with *T. gondii* tachyzoites of the ME49 strain and analyzed for parasite differentiation (A) Immunofluorescence microscopy of infected astrocytes cultured at neutral pH (7.2; 1st panel) or alkaline pH (8.0; 2nd, 3rd and 4th panel) for 2 ,4, and 8 days (tachyzoites/SAG1 stained = red, bradyzoites/BAG1 stained = green, nuclei/DAPI stained = blue). (B) Quantification of *T. gondii* BAG1 gene expression in infected WT and CD73$^{-/-}$ astrocytes normalized to host housekeeping gene PGK1. (C) Visualization and quantification of *in vitro* cyst formation in cultured WT and CD73$^{-/-}$ astrocytes after 8 d at pH 8.0 as determined by anti-BAG1 (green) and *Dolichos biflorus* agglutinin (DBA, red) staining; insets show cysts at higher magnification. Significant differences based on two-tailed t-tests are displayed (** P<0.01).

We next asked whether adenosine receptor signaling was important in mediating bradyzoite cyst maturation by treating astrocytes with an adenosine analogue, 5'-N-ethylcarboxamido adenosine (NECA). NECA acts as a broad spectrum adenosine receptor agonist, but does not participate in the adenosine purine salvage pathway. Interestingly, we found that NECA had no effect on cyst formation (Figure 2.9B). To confirm that the differentiation defect in CD73$^{-/-}$ cells was due to the absence of CD73 activity, we cultured astrocytes from WT and CD73$^{-/-}$ mice with ME49 tachyzoites in the presence of the specific CD73 inhibitor alpha,beta-methylene ADP (meADP) which blocks the enzymatic activity of CD73. As anticipated, meADP had no effect on cyst formation in CD73$^{-/-}$ astrocytes, whereas it significantly inhibited cyst formation in WT astrocytes (Figure 2.9B).

Finally, to determine whether CD73 expression on other cell types also promoted *T. gondii* cyst formation, we infected the human fibroblast cell line Hs27 (which is commonly used to maintain *T. gondii* tachyzoites) with *T. gondii* ME49 under cyst-inducing conditions. Since the cell-line expresses CD73 (Figure 2.9C), we were able to show that inhibition of CD73 enzymatic activity with meADP led to a 2-fold reduction in cysts generated under high pH (Figure 2.9D), similar to what we observe with primary mouse astrocytes (Figure 2.9B). Consistent with this finding, mice lacking the A_1 or A_{2A} adenosine receptors exhibited no defect in bradyzoite cyst formation, as their cyst burden was similar that of WT mice (Figure 2.9E). All together, these results confirm that CD73 promotes *T. gondii* bradyzoite differentiation through a mechanism involving adenosine acquisition rather than adenosine receptor signaling.

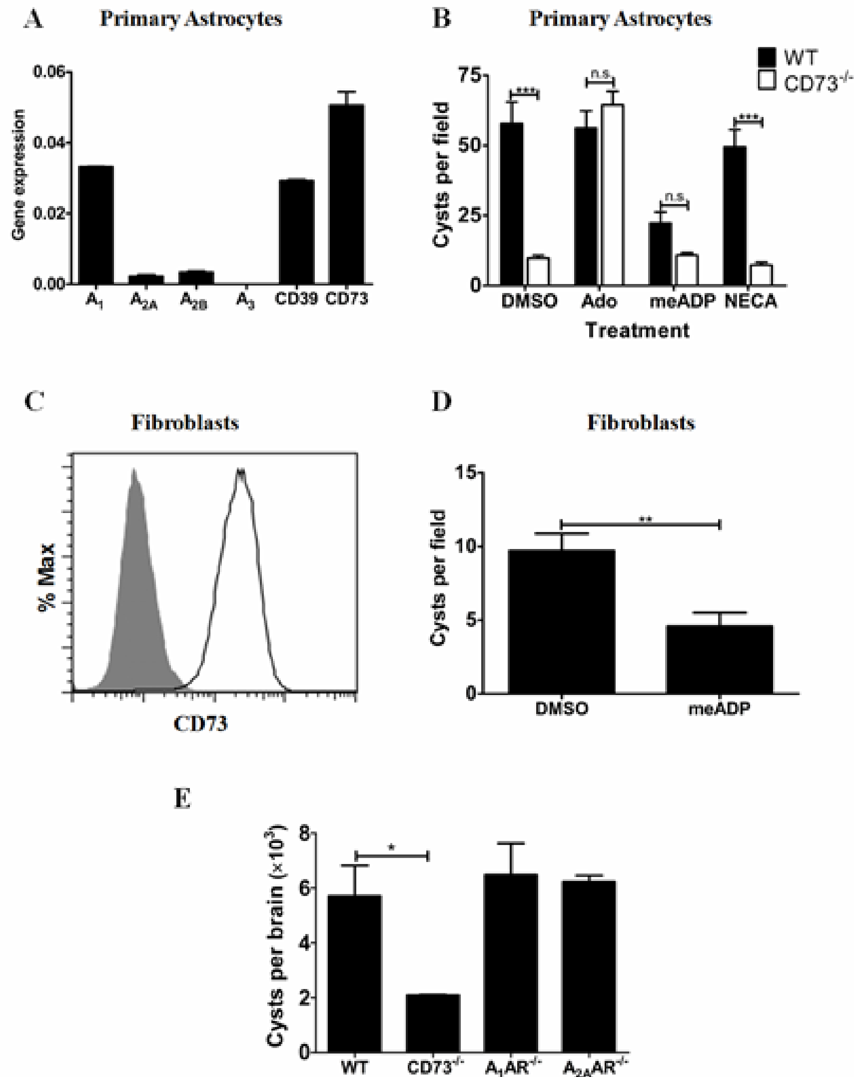


Figure 2.9. Exogenous adenosine rescues *T. gondii* cyst formation in CD73-deficient host cells independent of adenosine receptor signaling. (A) Gene expression analysis of adenosine receptors (A1, A2A, A2B, A3), CD39 and CD73 on cultured astrocytes. Real-time quantitative PCR results are normalized to the house keeping gene PGK1. (B) Astrocytes from WT and *cd73*$^{-/-}$ neonates were cultured and infected with *T. gondii* tachyzoites of the ME49 strain in the presence or absence of adenosine (50 µM), the broad spectrum adenosine receptor agonist NECA (10 µM), or the CD73 inhibitor alpha,beta-methylene ADP (meADP, 50 µM). Cyst formation was assessed 12 days after continuous culture at pH 8.0 by immunofluorescence

microscopy to quantify *Dolichos biflorus* agglutinin (DBA) binding cysts. (C) The human foreskin fibroblast line Hs27 was assessed for CD73 expression by flow cytometry (unshaded curve, CD73-PE labeled cells, shaded curve, unstained live cells). (D) Cyst formation was quantified as described above following 8 days' culture of *T. gondii*-infected Hs27 fibroblasts at pH 8.0 in the presence of DMSO or 50 µM meADP. Significant differences based on two-tailed t-tests are displayed (** $P<0.01$, *** $P<0.001$). (E) Quantitation of brain parasite cyst burden in $A_1AR^{-/-}$, $A_{2A}AR^{-/-}$, $CD73^{-/-}$ and WT mice infected with 20 cysts of *T. gondii* ME49 for 30 days (* $P<0.05$)

DISCUSSION

The ability of *Toxoplasma gondii* to infect a wide variety of warm-blooded hosts and virtually any nucleated cell poses a challenge in the control of this parasite. One reason that *T. gondii* is a successful parasite is its ability to differentiate to the slow-growing bradyzoite stage in skeletal muscle and the CNS, allowing the parasite to persist even in the face of a robust immune response (46, 47). In this study, we established that the widely expressed host-derived enzyme, 5'-ecto-nucleosidase (CD73), plays a significant role in *T. gondii* persistence during chronic infection. In mice infected with *T. gondii*, we observed a significant increase in CD73 expression in the brain, the major tissue parasitized during the chronic stage of infection. Importantly, $CD73^{-/-}$ mice were resistant to cerebral toxoplasmosis, with increased survival and reduced parasite loads in the brains of infected animals. This protection was observed despite similar kinetics of *T. gondii* dissemination into the brains of WT and $CD73^{-/-}$ mice, suggesting that CD73 expression promotes *T. gondii* persistence or survival *in situ*.

In the CNS, control of *T. gondii* infection is mediated by infiltrating leukocytes, particularly cytotoxic T lymphocytes that activate parasiticidal and parasitistatic mechanisms in

infected cells (6, 8, 26, 48). Recrudescence of infection occurs when there is a breakdown in immunosurveillance or in the absence of key mediators of parasite control, such as IL-12, IFNγ, iNOS, and TNF-□ (6). In $CD73^{-/-}$ mice, protection from cerebral toxoplasmosis was not associated with elevated IFNγ or other effector molecules such as iNOS and TNF-□ in the infected CNS. Moreover, the lack of CD73 on CNS resident cells did not alter *T. gondii*'s ability to proliferate.

While *T. gondii* can synthesize pyrimidines de novo, it lacks the ability to synthesize purines, including adenosine (20). CD73 mediates the final rate-limiting step in the enzymatic chain that catalyzes the dephosphorylation of extracellular AMP to adenosine from ATP. High levels of adenosine in the extracellular space trigger its transport into cells by means of transporters (49). The transport of adenosine across cell membranes is the first step in the salvage of adenosine by *T. gondii* (50-52). Although deletion of the major adenosine salvage enzyme adenosine kinase (AK) alone is not lethal and only imparts a modest growth defect (21), whether deletion of AK impairs cyst formation has not been reported . It is possible that under conditions where extracellular adenosine levels are diminished or absent such as in mice or cells lacking CD73, that there would be an overall deficit in the availability of adequate sources of adenosine. Such a deficit can have an impact on *T. gondii*'s ability to undergo efficient transformation to the bradyzoite or long-lived cyst stage. Thus, interference with adenosine uptake can be detrimental to *T. gondii*, as we observed in $CD73^{-/-}$ mice, and in *in vitro* differentiation assays in glial and fibroblast cells expressing or lacking CD73.

In summary, we demonstrated that $CD73^{-/-}$ mice are protected from *T. gondii* induced mortality and morbidity. Ablation of CD73 renders *T. gondii* incapable of efficiently establishing

latent infection as CD73$^{-/-}$ mice have dramatically decreased cyst burden than WT mice. We show that glial cells from CD73$^{-/-}$ mice are unable to sustain cyst differentiation or survival. While addition of adenosine rescues the cyst maturation defect, our results show this is not mediated by adenosine receptor signaling. Further, inactivation of CD73 enzymatic function with a pharmacological inhibitor significantly reduces cyst formation in WT astrocytes and human fibroblasts. Although the precise mechanism of how *T. gondii* may acquire host adenosine for its survival is not yet clear, these findings clearly indicate that inhibitors to CD73 or adenosine might be important targets for therapy in limiting the growth or survival of *Toxoplasma gondii* and other apicomplexan parasites.

REFERENCES

1. Kim K & Weiss LM (2004) Toxoplasma gondii: the model apicomplexan. *Int J Parasitol* 34(3):423-432.
2. Holland GN*, et al.* (1988) Ocular toxoplasmosis in patients with the acquired immunodeficiency syndrome. *Am J Ophthalmol* 106(6):653-667.
3. Falangola MF & Petito CK (1993) Choroid plexus infection in cerebral toxoplasmosis in AIDS patients. *Neurology* 43(10):2035-2040.
4. Lambert H & Barragan A (2010) Modelling parasite dissemination: host cell subversion and immune evasion by Toxoplasma gondii. *Cell Microbiol* 12(3):292-300.
5. Courret N*, et al.* (2006) CD11c- and CD11b-expressing mouse leukocytes transport single Toxoplasma gondii tachyzoites to the brain. *Blood* 107(1):309-316.
6. Daubener W & Hadding U (1997) Cellular immune reactions directed against Toxoplasma gondii with special emphasis on the central nervous system. *Med Microbiol Immunol* 185(4):195-206.
7. Gavrilescu LC & Denkers EY (2001) IFN-gamma overproduction and high level apoptosis are associated with high but not low virulence Toxoplasma gondii infection. *J Immunol* 167(2):902-909.
8. Wang X, Kang H, Kikuchi T, & Suzuki Y (2004) Gamma interferon production, but not perforin-mediated cytolytic activity, of T cells is required for prevention of toxoplasmic encephalitis in BALB/c mice genetically resistant to the disease. *Infect Immun* 72(8):4432-4438.
9. Yegutkin GG (2008) Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. *Biochim Biophys Acta* 1783(5):673-694.

10. Schetinger MR, Morsch VM, Bonan CD, & Wyse AT (2007) NTPDase and 5'-nucleotidase activities in physiological and disease conditions: new perspectives for human health. *Biofactors* 31(2):77-98.

11. Thompson LF*, et al.* (2004) Crucial role for ecto-5'-nucleotidase (CD73) in vascular leakage during hypoxia. *J Exp Med* 200(11):1395-1405.

12. Hasko G & Cronstein BN (2004) Adenosine: an endogenous regulator of innate immunity. *Trends Immunol* 25(1):33-39.

13. Okusa MD*, et al.* (2000) A(2A) adenosine receptor-mediated inhibition of renal injury and neutrophil adhesion. *Am J Physiol Renal Physiol* 279(5):F809-818.

14. Hasko G, Nemeth ZH, Vizi ES, Salzman AL, & Szabo C (1998) An agonist of adenosine A3 receptors decreases interleukin-12 and interferon-gamma production and prevents lethality in endotoxemic mice. *Eur J Pharmacol* 358(3):261-268.

15. Linden J (2001) Molecular approach to adenosine receptors: receptor-mediated mechanisms of tissue protection. *Annu Rev Pharmacol Toxicol* 41:775-787.

16. Grenz A*, et al.* (2007) Protective role of ecto-5'-nucleotidase (CD73) in renal ischemia. *J Am Soc Nephrol* 18(3):833-845.

17. Mills JH*, et al.* (2008) CD73 is required for efficient entry of lymphocytes into the central nervous system during experimental autoimmune encephalomyelitis. *Proceedings of the National Academy of Sciences of the United States of America* 105(27):9325-9330.

18. Carman AJ, Mills JH, Krenz A, Kim DG, & Bynoe MS (2011) Adenosine receptor signaling modulates permeability of the blood-brain barrier. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 31(37):13272-13280.

19. Blader IJ, Manger ID, & Boothroyd JC (2001) Microarray analysis reveals previously unknown changes in Toxoplasma gondii-infected human cells. *The Journal of biological chemistry* 276(26):24223-24231.

20. Schwartzman JD & Pfefferkorn ER (1982) Toxoplasma gondii: purine synthesis and salvage in mutant host cells and parasites. *Exp Parasitol* 53(1):77-86.

21. Chaudhary K*, et al.* (2004) Purine salvage pathways in the apicomplexan parasite Toxoplasma gondii. *J Biol Chem* 279(30):31221-31227.

22. Krug EC, Marr JJ, & Berens RL (1989) Purine metabolism in Toxoplasma gondii. *The Journal of biological chemistry* 264(18):10601-10607.

23. Denkers EY, Caspar P, Hieny S, & Sher A (1996) Toxoplasma gondii infection induces specific nonresponsiveness in lymphocytes bearing the V beta 5 chain of the mouse T cell receptor. *J Immunol* 156(3):1089-1094.

24. Conley FK & Jenkins KA (1981) Immunohistological study of the anatomic relationship of toxoplasma antigens to the inflammatory response in the brains of mice chronically infected with Toxoplasma gondii. *Infection and immunity* 31(3):1184-1192.

25. Suzuki Y, Orellana MA, Schreiber RD, & Remington JS (1988) Interferon-gamma: the major mediator of resistance against Toxoplasma gondii. *Science* 240(4851):516-518.

26. Sher A, Denkers EY, & Gazzinelli RT (1995) Induction and regulation of host cell-mediated immunity by Toxoplasma gondii. *Ciba Found Symp* 195:95-104; discussion 104-109.

27. Scharton-Kersten TM*, et al.* (1996) In the absence of endogenous IFN-gamma, mice develop unimpaired IL-12 responses to Toxoplasma gondii while failing to control acute infection. *J Immunol* 157(9):4045-4054.

28. Suzuki Y, Sa Q, Gehman M, & Ochiai E (2011) Interferon-gamma- and perforin-mediated immune responses for resistance against Toxoplasma gondii in the brain. *Expert Rev Mol Med* 13:e31.

29. Suzuki Y (2002) Host resistance in the brain against Toxoplasma gondii. *The Journal of infectious diseases* 185 Suppl 1:S58-65.

30. Scharton-Kersten TM, Yap G, Magram J, & Sher A (1997) Inducible nitric oxide is essential for host control of persistent but not acute infection with the intracellular pathogen Toxoplasma gondii. *The Journal of experimental medicine* 185(7):1261-1273.

31. Sibley LD, Adams LB, Fukutomi Y, & Krahenbuhl JL (1991) Tumor necrosis factor-alpha triggers antitoxoplasmal activity of IFN-gamma primed macrophages. *Journal of immunology* 147(7):2340-2345.

32. Tong N*, et al.* (2000) Neuronal fractalkine expression in HIV-1 encephalitis: roles for macrophage recruitment and neuroprotection in the central nervous system. *Journal of immunology* 164(3):1333-1339.

33. Deckert-Schluter M, Schluter D, Hof H, Wiestler OD, & Lassmann H (1994) Differential expression of ICAM-1, VCAM-1 and their ligands LFA-1, Mac-1, CD43, VLA-4, and MHC class II antigens in murine Toxoplasma encephalitis: a light microscopic and ultrastructural immunohistochemical study. *J Neuropathol Exp Neurol* 53(5):457-468.

34. Sasseville VG*, et al.* (1994) Monocyte adhesion to endothelium in simian immunodeficiency virus-induced AIDS encephalitis is mediated by vascular cell adhesion molecule-1/alpha 4 beta 1 integrin interactions. *The American journal of pathology* 144(1):27-40.

35. Steffen BJ, Butcher EC, & Engelhardt B (1994) Evidence for involvement of ICAM-1 and VCAM-1 in lymphocyte interaction with endothelium in experimental autoimmune encephalomyelitis in the central nervous system in the SJL/J mouse. *The American journal of pathology* 145(1):189-201.

36. Deckert M*, et al.* (2003) Mice with neonatally induced inactivation of the vascular cell adhesion molecule-1 fail to control the parasite in Toxoplasma encephalitis. *European journal of immunology* 33(5):1418-1428.

37. Wang X, Michie SA, Xu B, & Suzuki Y (2007) Importance of IFN-gamma-mediated expression of endothelial VCAM-1 on recruitment of CD8+ T cells into the brain during chronic infection with Toxoplasma gondii. *J Interferon Cytokine Res* 27(4):329-338.

38. Cotter R*, et al.* (2002) Fractalkine (CX3CL1) and brain inflammation: Implications for HIV-1-associated dementia. *J Neurovirol* 8(6):585-598.

39. Hughes PM, Botham MS, Frentzel S, Mir A, & Perry VH (2002) Expression of fractalkine (CX3CL1) and its receptor, CX3CR1, during acute and chronic inflammation in the rodent CNS. *Glia* 37(4):314-327.

40. Pfefferkorn ER & Pfefferkorn LC (1977) Specific labeling of intracellular Toxoplasma gondii with uracil. *J Protozool* 24(3):449-453.

41. Soete M, Fortier B, Camus D, & Dubremetz JF (1993) Toxoplasma gondii: kinetics of bradyzoite-tachyzoite interconversion in vitro. *Exp Parasitol* 76(3):259-264.

42. Bohne W, Gross U, Ferguson DJ, & Heesemann J (1995) Cloning and characterization of a bradyzoite-specifically expressed gene (hsp30/bag1) of Toxoplasma gondii, related to genes encoding small heat-shock proteins of plants. *Mol Microbiol* 16(6):1221-1230.

43. Ferguson DJ (2004) Use of molecular and ultrastructural markers to evaluate stage conversion of Toxoplasma gondii in both the intermediate and definitive host. *Int J Parasitol* 34(3):347-360.

44. Djurković-Djaković O (2001) Murine Model of Drug-induced Reactivation of Toxoplasma gondii. *Acta protozoologica* 40(2):99.

45. Soete M, Camus D, & Dubremetz JF (1994) Experimental induction of bradyzoite-specific antigen expression and cyst formation by the RH strain of Toxoplasma gondii in vitro. *Exp Parasitol* 78(4):361-370.

46. Sullivan WJ, Jr. & Jeffers V (2011) Mechanisms of Toxoplasma gondii persistence and latency. *FEMS Microbiol Rev*.

47. Camus D, Zalis MG, Vannier-Santos MA, & Banic DM (1995) The art of parasite survival. *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica ... [et al.]* 28(4):399-413.

48. Suzuki Y*, et al.* (2010) Removal of Toxoplasma gondii cysts from the brain by perforin-mediated activity of CD8+ T cells. *The American journal of pathology* 176(4):1607-1613.

49. Deussen A, Stappert M, Schafer S, & Kelm M (1999) Quantification of extracellular and intracellular adenosine production: understanding the transmembranous concentration gradient. *Circulation* 99(15):2041-2047.

50. Schwab JC, Afifi Afifi M, Pizzorno G, Handschumacher RE, & Joiner KA (1995) Toxoplasma gondii tachyzoites possess an unusual plasma membrane adenosine transporter. *Mol Biochem Parasitol* 70(1-2):59-69.

51. Chiang CW, *et al.* (1999) The adenosine transporter of Toxoplasma gondii. Identification by insertional mutagenesis, cloning, and recombinant expression. *J Biol Chem* 274(49):35255-35261.

52. De Koning HP, Al-Salabi MI, Cohen AM, Coombs GH, & Wastling JM (2003) Identification and characterisation of high affinity nucleoside and nucleobase transporters in Toxoplasma gondii. *International journal for parasitology* 33(8):821-831.

53. Gavrilescu LC & Denkers EY (2001) IFN-gamma overproduction and high level apoptosis are associated with high but not low virulence Toxoplasma gondii infection. *Journal of immunology* 167(2):902-909.

54. Dubey JP (1998) Refinement of pepsin digestion method for isolation of Toxoplasma gondii from infected tissues. *Veterinary parasitology* 74(1):75-77.

55. Fux B, *et al.* (2007) Toxoplasma gondii strains defective in oral transmission are also defective in developmental stage differentiation. *Infection and immunity* 75(5):2580-2590.

56. Aloisi F, Agresti C, & Levi G (1988) Establishment, characterization, and evolution of cultures enriched in type-2 astrocytes. *J Neurosci Res* 21(2-4):188-198.

CHAPTER 3

CD73-GENERATED ADENOSINE IS CRITICAL FOR IMMUNE REGULATION DURING *TOXOPLASMA GONDII* INFECTION

Deeqa A. Mahamed, Margaret S. Bynoe

ABSTRACT

As an obligate intracellular pathogen, the apicomplexan parasite *Toxoplasma gondii* evades immune system-mediated clearance by undergoing stage differentiation to persist indefinitely in susceptible hosts. Previously, we found that mice deficient in the ecto-enzyme CD73, which generates adenosine in the extracellular matrix, were significantly resistant to acute and chronic toxoplasmosis after oral infection with the *T. gondii* type II ME49 strain. Resistance in CD73-knockout mice was due to a delay in parasite differentiation in the CNS. To further clarify the role of CD73 and extracellular adenosine in *T. gondii* pathogenesis, we infected wildtype and CD73KO mice with ME49 cysts systemically by the intraperitoneal (IP) route. In contrast to oral infection, IP-infected CD73KO mice were highly susceptible to immune-mediated pathology, with significantly increased infiltration of neutrophils and T cells into the peritoneal cavity. Administration of the adenosine receptor agonist NECA to infected $CD73^{-/-}$ mice protected them against immunopathology, suggesting absence of CD73-generated adenosine led to the increased susceptibility in these mice. Peritoneal exudate cells from infected CD73KO mice generated higher levels of the inflammatory mediators nitric oxide, TNFα, and IL1β, without enhanced parasite killing or clearance. Bone marrow chimeras established that CD73 expression on both hematopoietic and nonhematopoietic compartments contributes to limiting *T. gondii*-induced immunopathology. In addition, mice deficient in the adenosine receptor A_{2A} were more susceptible to immunopathology during intraperitoneal infection with *T. gondii*, compared to wildtype mice. Thus extracellular adenosine is a key molecule that regulates the immune response to an intracellular pathogen and promotes host survival.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan pathogen with a broad host range and tissue trophism. During infection, *T. gondii* tachyzoites invade host cells and replicate rapidly before inducing a potent immune response that controls parasite numbers through direct killing. The immune response is dominated by IL-12 priming and subsequent IFNγ release by both innate and adaptive immune cells. Although a robust immune response is critical for controlling parasite-induced pathology, the immune response itself can cause severe pathology, and this balance may underscore differences in genetic susceptibility to toxoplasmosis among different hosts.

CD73, present on vertebrate host cells but not *T. gondii* parasites, is a surface-anchored glycoprotein that catalyses the conversion of AMP to adenosine, which is then sensed by the cell through transmembrane adenosine receptors. The enzyme is highly expressed on endothelial cells, epithelial cells and lymphocytes. Extracellular adenosine is associated with regulation of local inflammation. Indeed, mice deficient in CD73 exhibit several defects in immunoregulation following diverse inflammatory challenges. $CD73^{-/-}$ mice are more prone to sepsis (1), DSS-induced inflammatory bowel disease (2), lung injury (3), and tissue ischemia, hypoxia and inflammation (4-6). On the other hand, deletion or inhibition of CD73 may have therapeutic potential in cancer(7-9), multiple sclerosis (10) and chronic *Toxoplasma gondii* infection (11). Thus defining the role of this pleiotropic enzyme in inflammation and immunity may offer promising therapeutic targets.

In this study, we identify the critical role of CD73 in regulating the immune response to *T. gondii* infection in an acute systemic model of toxoplasmosis.

MATERIALS AND METHODS

Mice

C57Bl/6 mice were purchased from Jackson Laboratories. $CD73^{-/-}$ mice were obtained from Dr. Linda Thompson (OMRF). A1 and A2A mice were obtained from Dr. Jurgen Schnermann (NIH/NIDDK, Bethesda, MD) and Dr. Jiang-Fan Chen (Boston University School of Medicine, Boston, MA), respectively. Mice were bred and housed under specific pathogen-free conditions in the animal facility at Cornell University. All procedures performed on mice were approved by the Cornell University animal review committee.

Toxoplasma gondii infection

Wildtype (C57Bl/6, WT), $CD73^{-/-}$ $A_1AR^{-/-}$, and $A_{2A}AR^{-/-}$ mice were infected with 10, 20, or 40 *T. gondii* ME49 cysts or 1000 RH tachyzoites by intraperitoneal (ip) injection in 0.2 mL PBS. Survival rates and weight loss were monitored daily. In some experiments, groups of mice were euthanized at 3, 6, and 9 days post-infection and tissues (spleen, MLNs, liver, pancreas, lungs, and brains), peritoneal exudate cells, and/or blood collected for further processing.

Histology

Terminally anesthetized mice were perfused with ice-cold PBS and tissues collected for histology. Epidydymal fat pads were fixed in 10% formalin and processed for histology

Treatment with adenosine receptor agonists and antagonists

Mice infected as described above with 20 ME49 cysts were treated the adenosine analogue NECA. 5'-N-ethylcarboxamido adenosine (Tocris Biosciences, Minneapolis, USA) was

solubilized in DMSO and resuspended in PBS and was injected into CD73$^{-/-}$ IP daily (starting 3 days before infection) at a 200 µM concentration. The same final concentration of DMSO was used as vehicle controls.

Isolation of Peritoneal exudate cells

Naïve and infected mice were euthanized by inhalation of isoflurane prior to cell collection. Eight milliliters of ice-cold PBS was injected into the peritoneal cavity of each mouse, followed by 2 minutes palpitation to dislodge adherent peritoneal cells. The cells were then collected by aspiration with a syringe. Peritoneal cells were centrifuged at 400× g for 5 minutes and resuspended in complete RPMI (Cellgro) containing 10% v/v fetal calf serum, 1 mM Penicillin-Streptomycin, 25 mM HEPES, 1 mM L-glutamine and 50 µM beta-mercaptoethanol.

Isolation of spleen and mesenteric lymph node cells

Mesenteric lymph nodes and spleens were excised from euthanized mice and processed aseptically to generate single cell suspensions. Spleens were further treated to remove red blood cells using ACK lysing buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 100 µM EDTA). Cells were resuspended in complete RPMI media.

***Ex vivo* Flow cytometry**

Single cell suspensions were incubated with antibody cocktails in FACS buffer (0.5% BSA, 0.009% NaN3 in PBS) for 15 minutes at 4-8 °C. Antibody binding to the Fc receptor was blocked with 5 µg/mL rat anti-mouse CD16/CD32 antibody (FcBlock, BD Biosciences) prior to cell staining. Cells were washed, resuspended in FACS buffer and samples acquired on a FACS

Canto II instrument using FACSDiva software. Analysis of flow cytometry data was performed with Flowjo software.

Cytospins

Peritoneal exudate cells were labeled with CD45-APC and 1A8-FITC, then adhered to slides using a Cystospin. Cells were fixed with 2% paraformaldehyde, then mounted with Vectashield mounting media with DAPI. Slides were imaged with a Zeiss Axio Imager M1 fluorescent microscope.

Cell culture

Spleen, mesenteric lymph node and peritoneal exudate cells were resuspended to 5×10^6 per mL media and cultured at 37°C in a 5% CO_2 humidified incubator for 24 or 48 hours with or without soluble tachyzoite antigen (STAg). Cell-free supernatants were collected and stored at -20°C until analysis.

Cytokine ELISAs

Cell culture supernatants and serum of naïve infected mice were analyzed for IL1beta, TNFalpha, IL12 and interferon gamma using Ready-SET-GO ELISA kits from eBioscience according to the manufacturer's instructions.

Measurement of nitrite production

Nitric oxide levels in serum and cell culture supernatants were quantified through measuring the presence of nitrite using the Griess Reagent(12). Briefly, an equal volume of cell culture supernatant was incubated with the Griess reagent (0.1% N-1-napthylethylenediamine

dihydrochloride pre-mixed with 1% sulfanilamide in 5% phosphoric acid) and read at 550 nm in a BioTek plate reader. A sodium nitrite standard curve was used to quantify nitrite levels.

Bone Marrow Chimeras

Wildtype and CD73-deficient mice were lethally irradiated to ablate bone marrow cells (450 rads twice 4 hours apart). Mice were then reconstituted with bone marrow cells from congenic wildtype or $CD73^{-/-}$ donor mice. Each mouse received 10^6 BM cells iv, and was placed on acidified water containing Bactrim for 10 days to reduce the risk of opportunistic infection. After 8-10 weeks, mice were infected with 20 *T. gondii* ME49 cysts ip.and monitored for survival and weight loss.

Adoptive transfer

$RAG1^{-/-}$ mice were reconstituted with 10^7 total spleen leukocytes per mouse from wildtype or $CD73^{-/-}$ hosts by retro-orbital injection. One week later, mice were infected with 20 ME49 cysts of *T. gondii* ip. and monitored for morbidity and mortality

RESULTS

Intraperitoneal infection with Toxoplasma gondii is lethal in $CD73^{-/-}$ hosts

Mice infected with T. gondii via the intraperitoneal route develop systemic inflammation, characterized by recruitment and activation of leukocytes in the peritoneal cavity, increased T_h1 cytokines in serum and release of acute phase mediators such as nitric oxide by responding cells. In the absence of CD73, we found a significant and lethal dysregulation of the immune response

to *T. gondii*, with increased morbidity and mortality in CD73$^{-/-}$ hosts (Figure 3.1) infected with both the avirulent type II (Figure 3.1A and B) ME49 strain, or the highly virulent type I strain (Figure 3.2). Indeed, gross pathology of the peritoneal cavity show extensive fatty tissue necrosis (Figure 3.1C). Histological sections of epidydemal fat pads showed acute inflammation and infiltration of cells in the infected CD73$^{-/-}$ mice only (Figure 3.1D), suggesting that *T. gondii* infection induced recruitment and/or uncontrolled activation of immune cells.

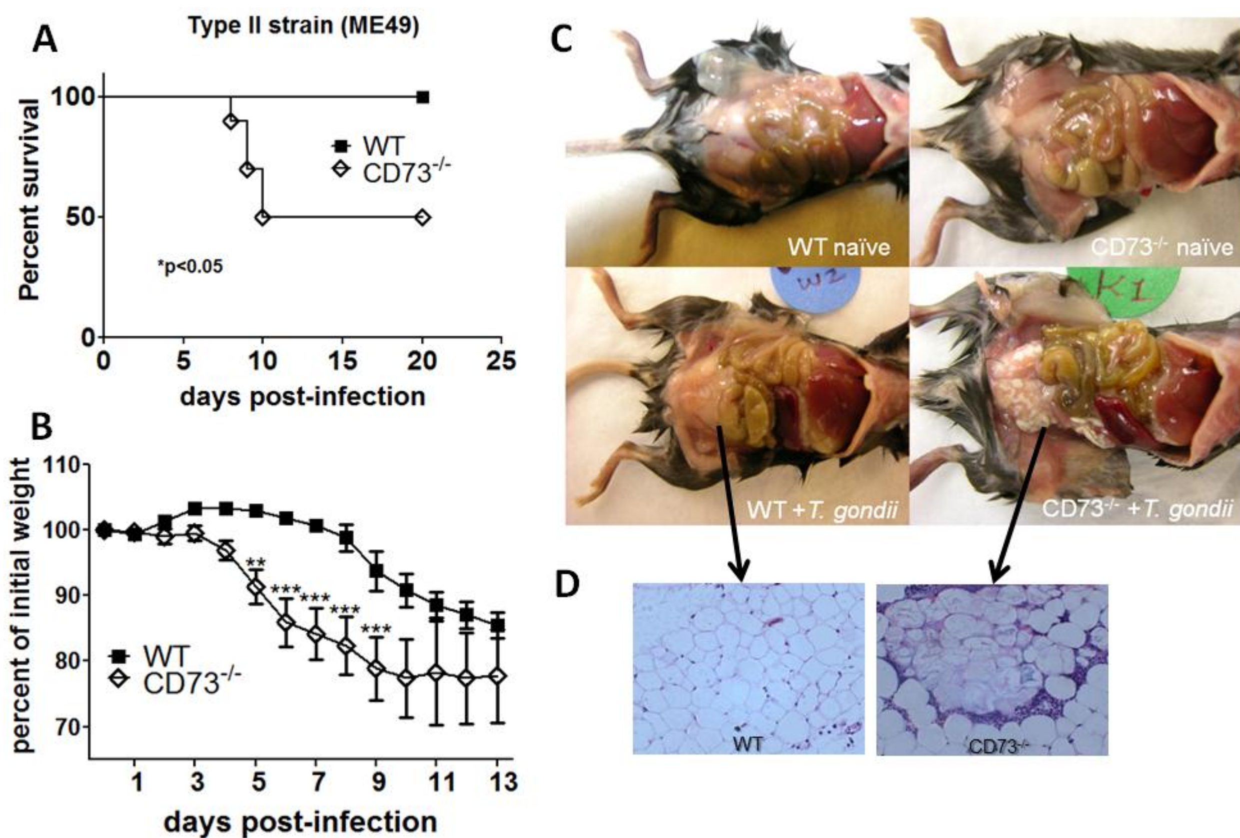


Figure 3.1. CD73$^{-/-}$ mice are more susceptible to acute toxoplasmosis compared to wild-type mice. CD73-knockout (CD73$^{-/-}$) mice and wildtype littermates were infected with *T. gondii* ME49 cysts by intraperitoneal injection. (A) Survival curve. (B) Weight loss (C) Gross pathology (D) H&E sections of epidydymal fat pads of infected mice. N=4-5 mice per group.

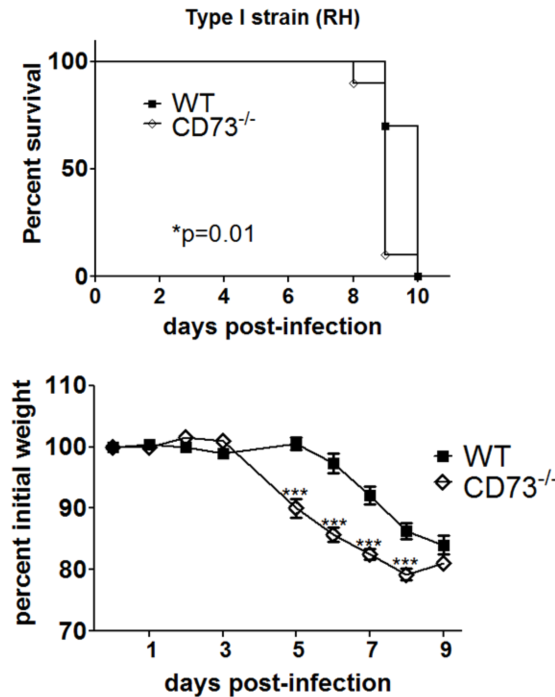


Figure 3.2. Intraperitoneal infection with *Toxoplasma gondii* RH is more lethal in CD73$^{-/-}$ hosts. A) Survival of mice infected with 1000 RH tachyzoites i.p. (B) Weight loss. *** $P < 0.001$, N=10 mice per group.

Treating CD73$^{-/-}$ mice with the adenosine receptor agonist NECA ablates susceptibility

Since CD73 has both enzymatic and signaling properties, we next determined which functional absence led to the susceptibility of CD73$^{-/-}$ mice to *T. gondii* infection. We infected WT and CD73$^{-/-}$ mice with T. gondii ME49 cysts i.p., and simultaneously treated a group of CD73$^{-/-}$ mice with the adenosine receptor analog 5'-N-ethylcarboxamido adenosine (NECA) and then monitored mice for weight loss and morbidity. As we observed previously, CD73$^{-/-}$ mice lost more weight and exhibited increased fatty tissue necrosis compared to wildtype mice (Figure 3.3). We found that NECA, which has a high affinity for adenosine receptors and mediates receptor signaling but does not participate in the purine salvage pathway, protected CD73$^{-/-}$ mice

from *T. gondii*-induced immunopathology compared to CD73$^{-/-}$ mice treated with DMSO only (Figure 3.3). This suggested that the susceptibility of CD73$^{-/-}$ hosts to *T. gondii* infection via the intraperitoneal route was due mainly to an absence of extracellular adenosine.

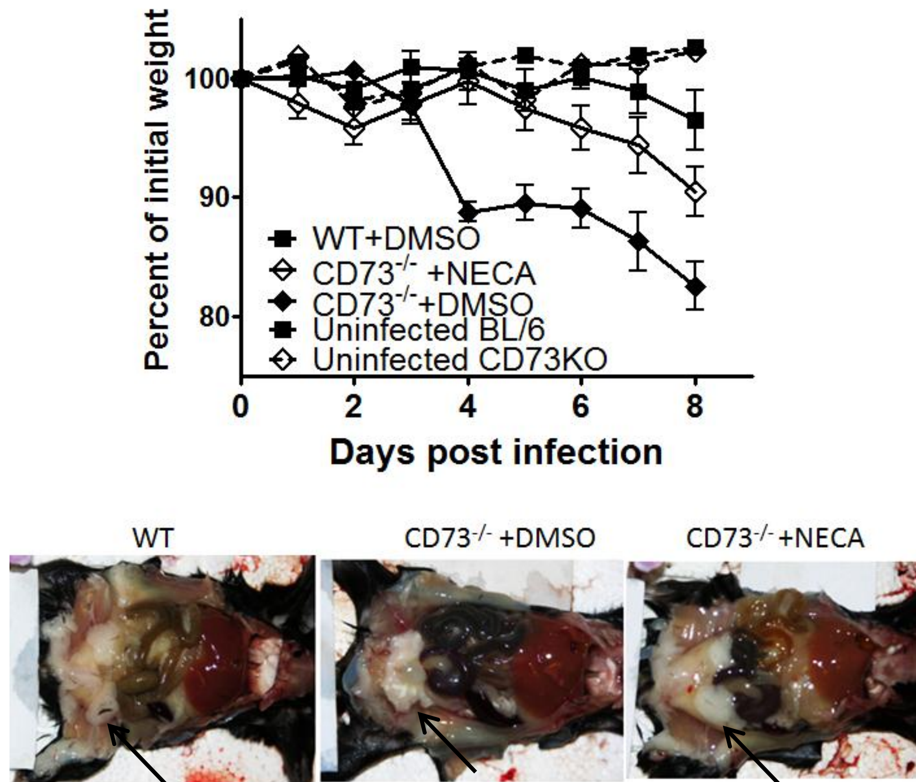


Figure 3.3. CD73-generated adenosine protects mice against inflammation via adenosine receptor signaling. CD73-deficient mice treated with the adenosine analog 5'-N-ethylcarboxamido adenosine (NECA) were protected from *T. gondii*-induced immunopathology compared to CD73$^{-/-}$ mice treated with DMSO only. (A) Weight loss (B) Gross pathology.

The A_{2A} adenosine receptor knockout mouse recapitulates the susceptibility to ip infection observed in CD73-deficient hosts

All four adenosine receptors have been implicated in modulating the immune response. In particular, the A_1 and A_{2A} receptors have been shown to regulate various aspects of

inflammation, from recruitment of leukocytes to effector functions of the innate and adaptive immune response. To determine which adenosine receptor played a dominant role in protection against *T. gondii*-induced immunopathology, we infected A_1AR and $A_{2A}AR$ knockout mice, and compared their susceptibility to WT and $CD73^{-/-}$ mice. We found that, while $A_1AR^{-/-}$ mice had a similar outcome of disease as wildtype mice, A2AAR-deficient mice mirrored $CD73^{-/-}$ mice in susceptibility (Figure 3.4). From this we concluded that the susceptibility of $CD73^{-/-}$ mice was due to the absence of A_{2A} adenosine receptor signaling.

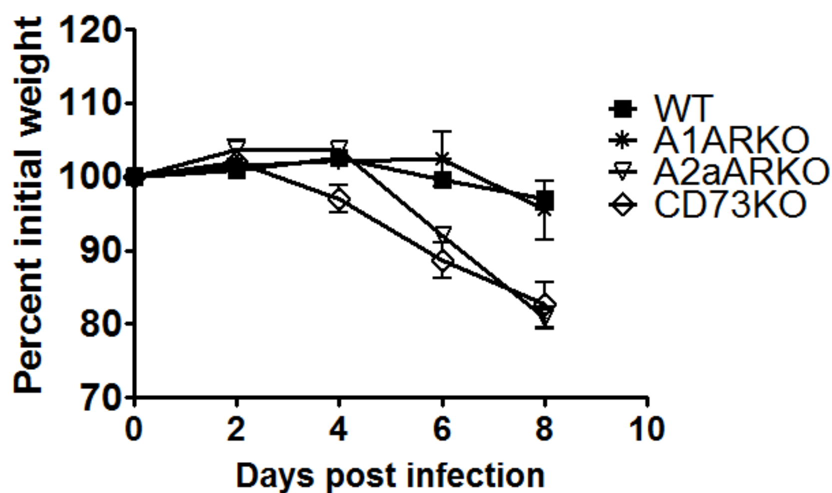


Figure 3.4. The A_{2A} adenosine receptor mediates the protective effects of extracellular adenosine. Wildtype, CD73KO, A_1 adenosine receptor (A_1ARKO) or A2A adenosine receptor (A_{2A}ARKO) knockout mice were infected with 20 ME49 cysts of *T. gondii* and monitored for weight loss.

Hematopoietic and nonhematopoietic cells contribute CD73-generated adenosine to limit inflammation

Next, we wanted to elucidate which cell-type was responsible for the generation of extracellular adenosine needed to limit T. gondii induced immunopathology. Cells of both hematopoietic and non-hematopoietic lineage express CD73. About a third of naïve CD45^{+} resident peritoneal cells express CD73, as determined by flow cytometry (Figure 3.5). The major cell population expressing CD73 is B cells, a quarter of which were positive as determined by flow cytometry (Figure 3.5). Both CD4^{+} and CD8^{+} T cells were mainly CD73^{+}, although overall there were few T cells in the peritoneal resident cell compartment.

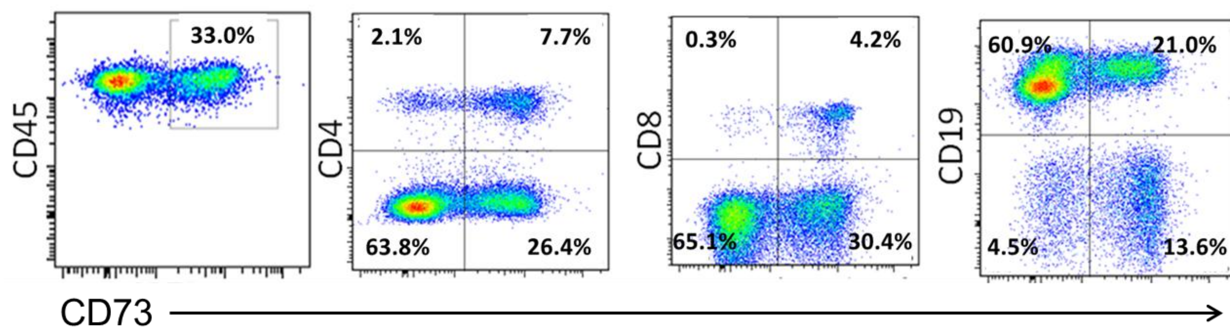


Figure. 3.5. Constitutive CD73 expression on resident peritoneal exudate cells. Naïve WT peritoneal cells were immunostained with antibodies to CD45, CD4, CD8, CD73 and CD19 and analyzed by flow cytometry.

Lymphocyte subsets and cells of the myeloid lineage are major sources of CD73-generated adenosine. Endothelial cells, epithelial cells and fibroblasts are all known to express CD73, and CD73-deficiency on endothelial cells has been shown to promote lymphocyte extravasation and accumulation at sites of inflammation. To determine the contributions of these two compartments to the critical release of CD73-derived extracellular adenosine, we generated

chimeric mice in which WT and CD73$^{-/-}$ mice were depleted of bone marrow cells through lethal irradiation, then rescued with the adoptive transfer of bone marrow cells from either WT or CD73$^{-/-}$ donors. The recipient mice all survived the irradiation and were reconstituted with hematopoietic cells of donor cells. After infecting these mice with *T. gondii* ME49, we monitored survival and weight loss. We found that all CD73$^{-/-}$ mice died by day 10 after infection, whether reconstituted with CD73^{+} or CD73^{-} bone marrow cells. This suggests that CD73-expression on the non-hematopoietic lineage is critical for preventing immunopathology induced by *T. gondii* systemic infection. Interestingly, WT mice reconstituted with CD73-deficient cells were partially susceptible, losing more weight and exhibiting increased mortality compared to WT mice reconstituted with CD73^{+} bone marrow cells. The mice succumbed later than CD73$^{-/-}$ mice, and 1 out of 5 mice survived the acute stage of disease. This suggests that early during infection, adenosine generated by nonhematopoietic cells is indispensable for controlling disease, which immune cell generated adenosine could be critical somewhat later during the acute stage of disease.

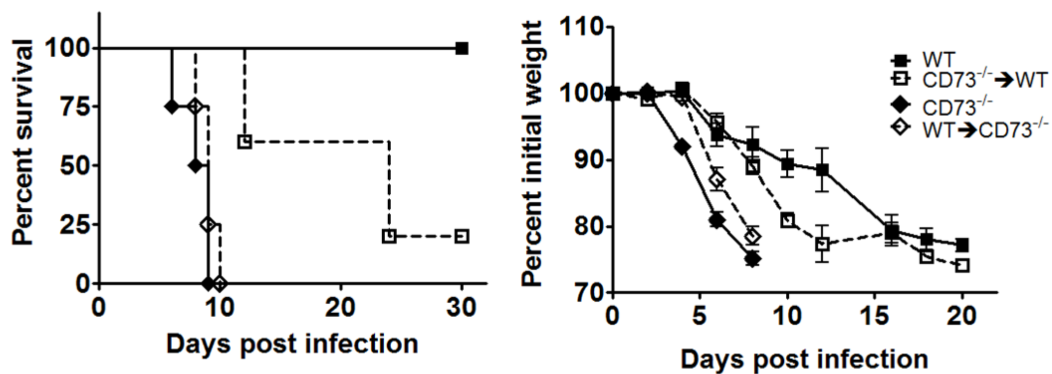


Figure 3.6. Hematopoietic and nonhematopoietic cells contribute CD73-generated adenosine to limit inflammation. Bone marrow chimeras were generated and infected with T. gondii ME49 cysts ip, then monitored for weight loss (A) and survival (B).

However, infection of RAG1$^{-/-}$ mice, which lack T and B lymphocytes, reconstituted with either wildtype or CD73$^{-/-}$ splenocytes before infection with *T. gondii* established that CD73 expression was not necessary on T or B cells (Figure 3.7) to mediate protection.

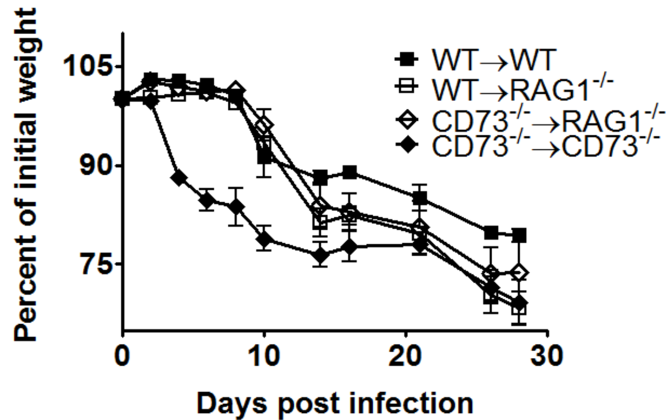


Figure 3.7. Absence of CD73 in the T or B cell compartment does not confer susceptibility to *T. gondii*. RAG1-knockout mice were reconstituted with splenocytes from WT or CD73$^{-/-}$ mice and compared in their susceptibility to *T. gondii* infection. WT mice were reconstituted with the same number of WT splenocytes, and CD73$^{-/-}$ mice with CD73$^{-/-}$ splenocytes.

Susceptibility is associated with increased and sustained infiltration of neutrophils and T lymphocytes

To determine the cause of the morbidity and mortality observed in the absence of CD73, we characterized the immune response to T. gondii i.p. infection in WT and CD73$^{-/-}$ hosts. *T. gondii* infection led to infiltration of CD45+ cells in the peritoneal cavity, with a steady increase in the absolute numbers of cells over the course of acute infection (Figure 3.8A). The composition of the peritoneal exudate cells changed dramatically during the course of infection. Resident PECs are mainly ~60% B cells, 20-30% macrophages, and 10-20% T cells. During the

course of infection, the proportion of B cells decreases, and neutrophils, T cells, and NK cells numbers increase dramatically both proportionally and in absolute numbers (Figure 3.8B, C)

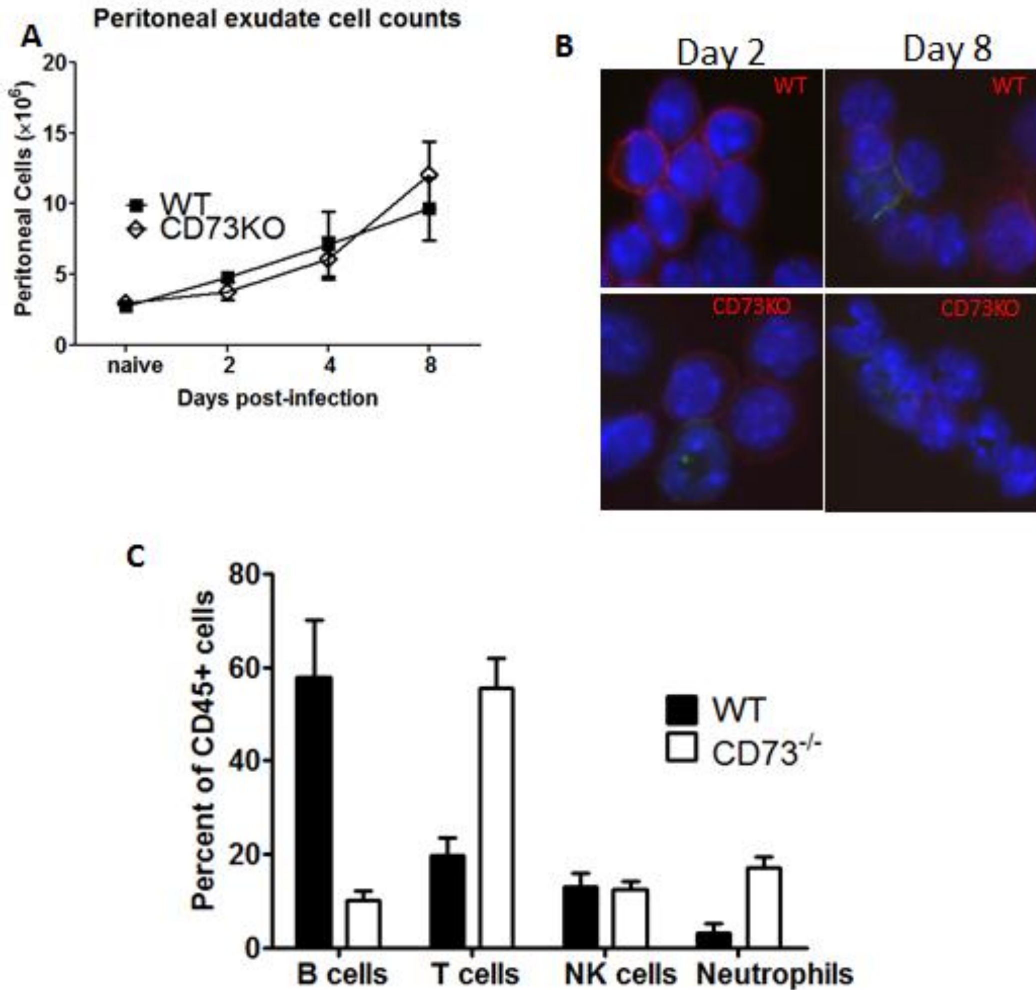


Figure 3.8. Cell composition comparison of peritoneal exudate cells (PECs) from infected wildtype and CD73KO mice. Cells were collected by peritoneal lavage from wildtype and CD73KO mice infected i.p. with 20 T. gondii ME49 cysts and stained for immune cell markers for flow cytometric analysis and cytospin. (A) Absolute PEC counts (B)Cytospins of cells stained for CD45 (pseudo-colored red) and 1A8 (neutrophils, pseudo-colored green) (C) Cell composition on day 8 as quantified by flow cytometry. N=3-4 per group per time-point.

Parasite control in CD73-deficient mice is limited.

The next question we wanted to address was whether the heightened immune response in CD73-deficient mice promoted better parasite control. We collected peritoneal exudate cells, splenic leukocytes, and mesenteric lymph node cells from mice at 2, 4, and 8 days post-infection and assessed parasite burden by flow cytometry. In the peritoneal exudate cells, both $CD11b^{+}$ and CD11b- cells were found to be harboring *T. gondii* tachyzoites during the acute stage of infection in both wildtype and $CD73^{-/-}$ mice (Figure 3.9). Interestingly, more cells from the mesenteric lymph nodes of CD73-deficient mice were infected with *T. gondii*, suggesting that the parasite was able to disseminate more systemically in these animals. Nevertheless, during chronic infection we found that surviving $CD73^{-/-}$ mice recovered and had reduced parasite burden in the brains (Figure 3.12), which we find consistent with our previous findings that CD73 promotes *T. gondii* persistence in the CNS during chronic infection.

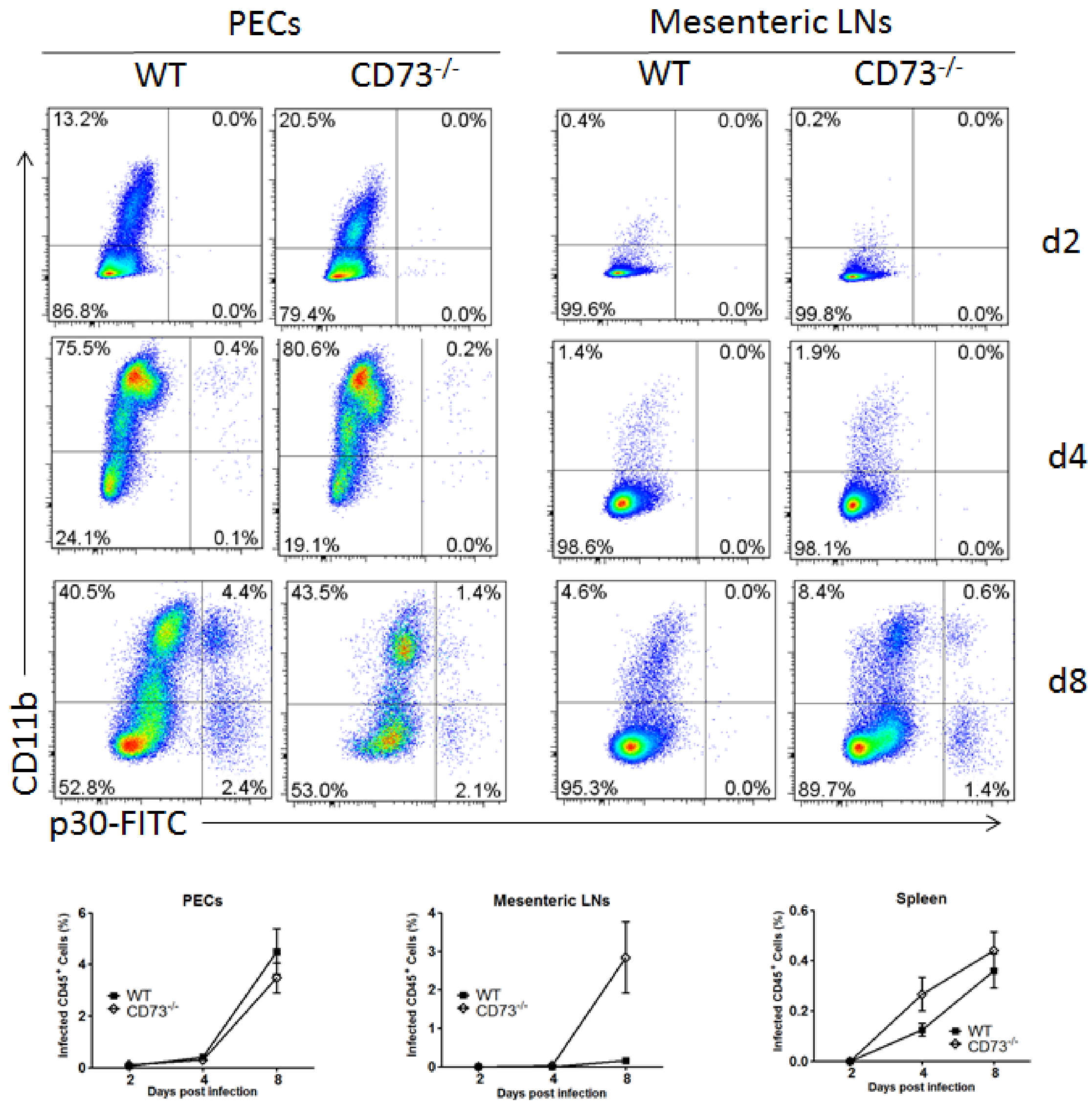


Figure 3.9. Parasite dissemination during acute infection. Mice were infected with 20 cysts of ME49 and euthanized 2, 4, or 8 days later. Peritoneal exudate cells mesenteric lymph node and spleen leukocytes were collected and stained for CD11b and *T. gondii* p30 to quantify parasitized cells by flow cytometry. N=3 per group per time point.

Increased inflammatory mediators in the absence of extracellular adenosine

The greatly perturbed cellular infiltration and immunopathology observed in *T. gondii* infected $CD73^{-/-}$ mice suggested the differences could be rooted dysregulated cytokine production. We thus analyzed the sera an cell culture supernatants of mice infected with T. gondii ip. We found that $CD73^{-/-}$ mice had elevated acute phase mediators such as IL1β, TNFα and increased nitric oxide (Figure 3.10 A-C). $CD73^{-/-}$ mice also exhibited elevated IFNγ in serum (Figure 3.10 E). Surprisingly, IL-12 was dramatically reduced in the sera of $CD73^{-/-}$ mice (Figure 3.10 D).

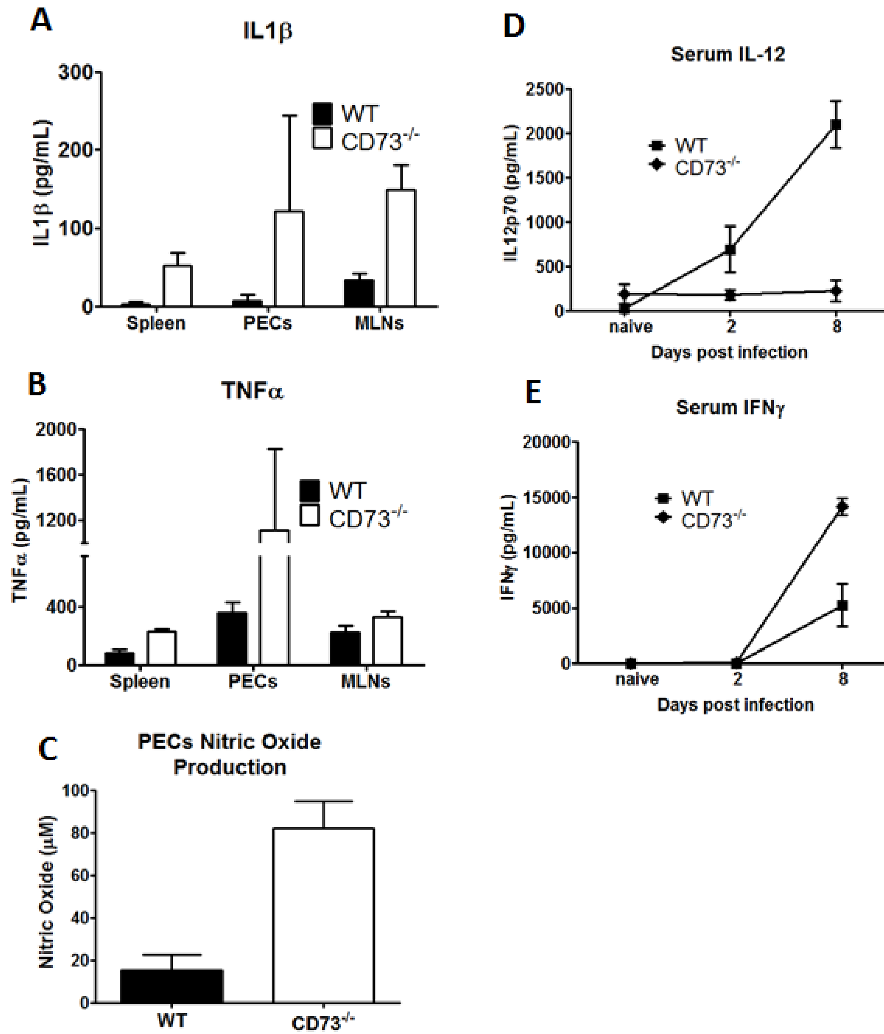


Figure 3.10. Dysregulated cytokine responses in CD73$^{-/-}$ mice. Spleen, peritoneal exudate or mesenteric lymph node cells from WT and CD73KO mice infected with 20 ME49 cysts for 8 days were cultured *in vitro* with *T. gondii* antigens (STAg, 20 µg/ml) for 48h then assayed for IL1-beta (A) or TNF-alpha (B) production by ELISA. (C) Peritoneal exudate cell nitric oxide production as measured with the Griess reagent. (C) Serum IL-12 (D) Serum interferon gamma.

In further experiments, we found that IL-12 production was significantly depressed in CD73$^{-/-}$ mice whether infected with live parasites or stimulated with *T. gondii* soluble antigens

(STAg) (Figure 3.10). Despite the reduced IL-12, STAg alone could recruit elevated neutrophils in the absence of CD73 (Figure 3.11).

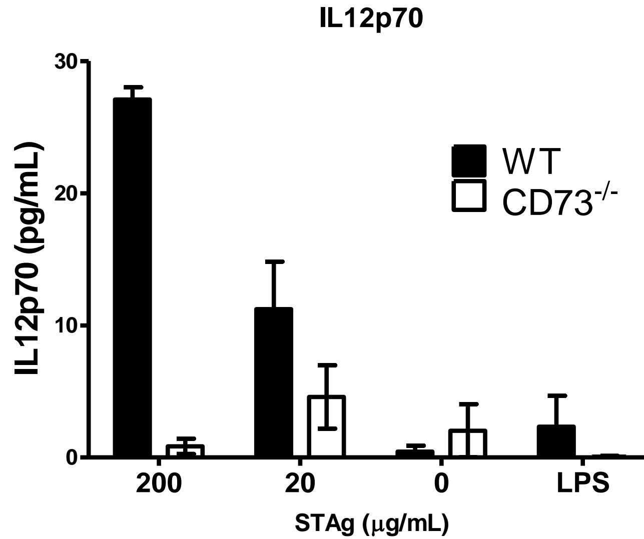


Figure 3.11. Reduced IL-12 secretion by peritoneal exudate cells from CD73$^{-/-}$ mice. Naïve wildtype and CD73$^{-/-}$ mice were injected i.p. with 20 ug/ml STAg or 1 ug/ml LPS in PBS followed by isolation of PECs and restimulation with STAg or LPS *in vitro* (n=3 per group).

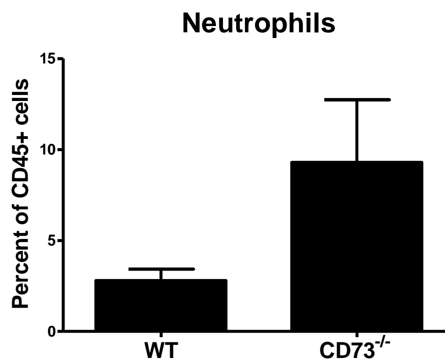


Figure 3.12. Increased neutrophil recruitment into the peritoneal exudate cell compartment in CD73$^{-/-}$ mice after sTAg treatment. WT and CD73$^{-/-}$ were injected with 25 ug of STAg ip.

PECs were collected 2 h later and analyzed by flow cytometry for neutrophil recruitment ($1A8^{+}CD45^{+}$). N= 3 mice per group.

$CD73^{-/-}$ mice retain a moderate resistance to chronic *T. gondii* infection despite early acute susceptibility

We next wondered if the enhanced susceptibility observed in response to acute intraperitoneal infection in $CD73^{-/-}$ hosts was retained during chronic infection, despite our earlier finding of resistance in this strain to oral infection. We found that $CD73^{-/-}$ mice did not show increased susceptibility to chronic infection if they survived the acute stage of disease (Figure3.12) as measured by cyst burden in the brain or morbidity. These results are in agreement with those we previously observed in peroral infection (11), and confirm the importance of CD73 for *T. gondii* persistence in the CNS.

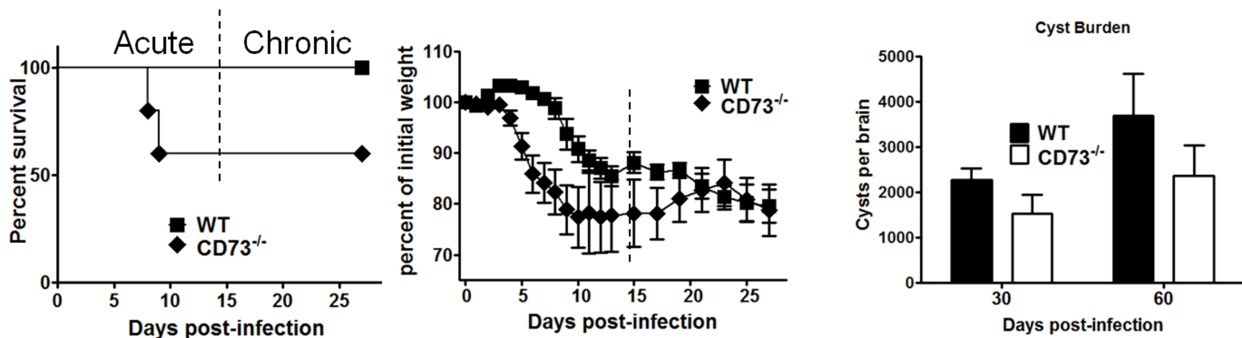


Figure 3.12. $CD73^{-/-}$ mice do not have increased susceptibility to chronic infection. Mice infected with T. gondii ip were monitored for survival and weight loss. (A) Survival (B) Weight loss. (C) Cyst counts per brain in surviving mice.

DISCUSSION

Infection with *T. gondii* via intraperitoneal injection induces rapid immune cell infiltration and a robust immune response in mice. The genetics of both the host and parasite influence the outcome of infection. The immune response is dominated by a pro-inflammatory, Th1 skewed response that is both critical for resistance and also detrimental to the host if not tightly regulated. In the current study, we found that the ectoenzyme CD73 was critical for limiting immunopathology and survival of mice infected with *T. gondii* via the intraperitoneal route. The protective effects of CD73 were mediated by the generation of extracellular adenosine, which prevented a pro-inflammatory cascade. We found that the A_{2A} adenosine receptor was the major receptor implicated in protecting mice against hyper-inflammatory cellular infiltration in CD73-deficient mice. These cells also secreted higher levels of proinflammatory mediators such as IL1β, TNF-α and nitric oxide, all of which are known to mediate bystander immunopathology. Thus the cytokine dysregulation we observed in the absence of CD73 may promote tissue pathology during the acute response.

In the absence of CD73, mice may exhibit a pro-inflammatory phenotype as a consequence of either lack of adenosine directly, or indirectly through the buildup of ATP, which is known to act as a danger signal. Our data indicates that the adenosine receptor A_{2A} is largely responsible for the limiting effect of adenosine in systemic inflammation induced by *T. gondii* ip infection.

In conclusion, in this study we identified a host genetic factor that may be implicated in controlling immune cell-mediated pathology associated with a protozoan infection.

REFERENCES

1. Hasko G, *et al.* (2011) Ecto-5'-nucleotidase (CD73) decreases mortality and organ injury in sepsis. *Journal of immunology* 187(8):4256-4267.
2. Bynoe MS, Waickman, A., Mahamed, D.A., Mueller, C., Mills, J.H., Czopik, A. (2012) CD73 Is Critical for the Resolution of Murine Colonic Inflammation. *Journal of Biomedicine and Biotechnology* 2012:13.
3. Volmer JB, Thompson LF, & Blackburn MR (2006) Ecto-5'-nucleotidase (CD73)-mediated adenosine production is tissue protective in a model of bleomycin-induced lung injury. *J Immunol* 176(7):4449-4458.
4. Thompson LF, *et al.* (2004) Crucial role for ecto-5'-nucleotidase (CD73) in vascular leakage during hypoxia. *J Exp Med* 200(11):1395-1405.
5. Blume C, *et al.* (2012) Autoimmunity in CD73/Ecto-5'-nucleotidase deficient mice induces renal injury. *PLoS One* 7(5):e37100.
6. Jian R, *et al.* (2012) CD73 protects kidney from ischemia-reperfusion injury through reduction of free radicals. *APMIS* 120(2):130-138.
7. Salmi M & Jalkanen S (2012) Host CD73 impairs anti-tumor immunity. *Oncoimmunology* 1(2):247-248.
8. Zhang B (2012) CD73 promotes tumor growth and metastasis. *Oncoimmunology* 1(1):67-70.
9. Yegutkin GG, *et al.* (2011) Altered purinergic signaling in CD73-deficient mice inhibits tumor progression. *Eur J Immunol* 41(5):1231-1241.

10. Mills JH, *et al.* (2008) CD73 is required for efficient entry of lymphocytes into the central nervous system during experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* 105(27):9325-9330.

11. Mahamed DA, Mills JH, Egan CE, Denkers EY, & Bynoe MS (2012) CD73-generated adenosine facilitates Toxoplasma gondii differentiation to long-lived tissue cysts in the central nervous system. *Proc Natl Acad Sci U S A* 109(40):16312-16317.

12. Green LC, *et al.* (1982) Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal Biochem* 126(1):131-138.

CHAPTER 4

SUMMARY AND CONCLUSIONS

There are quite a few potential points of interaction between CD73, extracellular adenosine and *Toxoplasma gondii* pathogenesis. Firstly, when *T. gondii* cysts are ingested by the host, the liberated bradyzoites differentiate to tachyzoites and invade the epithelial cells lining the small intestine epithelium. These cells express CD73, and can respond to adenosine receptor signaling (1, 2). Secondly, dissemination of *T. gondii* to other tissues requires trafficking through barriers such as the epithelium of the small intestine, the endothelium of blood vessels, and the blood brain barrier. Adenosine receptor signaling has been shown to modulate the integrity of all of these biological barriers. In addition, the immune response to *T. gondii* is a critical component of the pathogenesis of this parasite, and adenosine regulates many aspects of both innate and adaptive immunity, such as cell migration, activation, cytokine production and resolution of the immune response. Finally, unlike vertebrate cells which are capable of *de novo* adenosine synthesis, *T. gondii* must rely solely on the purine salvage pathway, necessitating the presence of host-generated adenosine within host cells. Limiting access to adenosine may thus affect *T. gondii* survival or virulence, as the nucleoside is an important intermediary for many metabolic and signaling pathways.

To determine the role of CD73 in *T. gondii* infection, we infected wildtype and CD73-knockout mice with the *T. gondii* ME49 strain via the oral route. We found that while CD73$^{-/-}$ mice were protected from acute *T. gondii* oral infection, the most significant impact of CD73 was in the CNS, with strikingly attenuated chronic stage infection. CD73$^{-/-}$ mice did not succumb to reactivation of infection, and developed fewer brain cysts.

One possibility for the attenuated chronic infection in CD73$^{-/-}$ hosts could be due to differences in the early kinetics of parasite dissemination. We found that *T. gondii* proliferation and dissemination between wildtype and CD73-deficient hosts was similar early during

infection. The parasite initially expanded in the small intestinal ileum, then was detectable in the spleen, liver and lungs, all of which saw a peak in parasite burden 7-10 days post-infection, before parasite numbers were curtailed efficiently. In contrast, *T. gondii* was detectable in the brain a little later than in the viscera, but continued to expand without a significant contraction in numbers. Instead, analysis of parasite differentiation genes show a major shift to bradyzoites and histological evidence confirm parasite cysts by one month after infection.

Interestingly, in comparison to wildtype C57BL/6 mice, the reduced cyst burden in CD73$^{-/-}$ mice was directly attributable to a delay or defect in tachyzoite-to-bradyzoite differentiation, which we define by a reduction in tachyzoite-specific SAG1 expression and a concomitant increase in bradyzoite specific BAG1 gene expression in the brain. Analysis of IFNγ, iNOS and TNFα gene expression by qRT-PCR revealed that CD73$^{-/-}$ and WT mice had similar levels of these immune response genes known to inhibit *T. gondii* proliferation in the brain. This suggested that CD73 could promote *T. gondii* bradyzoite differentiation directly. To test this hypothesis, we isolated astrocytes from WT and CD73$^{-/-}$ hosts and infected them with tachyzoites *in vitro*. Astrocytes have been shown to harbor cysts *in vivo*, and have been shown to be permissive to *T. gondii* differentiation in vitro under cyst-inducing conditions. We found that the CD73-dependent difference in *T. gondii* differentiation was maintained in astrocytes. In fact, after 8 days in culture, tachyzoites spontaneously expressed bradyzoite BAG1 mRNA in WT astrocytes. Under high pH stress, bradyzoites fully differentiated to mature cysts, as defined by the detection of DBA+ BAG1+ cysts. Few cysts formed in CD73-/- astrocytes under the same conditions, which led us to conclude that CD73 promoted *T. gondii* cyst formation in astrocytes. Further in vitro and in vivo experiments revealed that adenosine, rather than adenosine receptor signaling on astrocytes, was responsible for the robust cyst formation observed in CD73+

astrocytes. The phenomena could be confirmed in the human foreskin fibroblast cell line Hs27, which led us to conclude that CD73-generated adenosine directly promoted *T. gondii* cyst formation or persistence.

Although the mechanism by which CD73 enhances *T. gondii* cyst formation is unclear, several possibilities present themselves. One intriguing possibility is that CD73 is incorporated into the parasitophorous vacuole membrane, which is derived from the host cell plasma membrane. Since CD73 is a GPI-anchored protein, it may not be excluded from the nascent PV as *T. gondii* invades the host cell (3). Whether it is in fact incorporated into the PVM is difficult to ascertain, since antibodies to CD73 failed to recognize the protein after cell/tissue fixation. However, it is interesting that *T. gondii* expresses a highly active apyrase which, similar to CD39, generates AMP from ATP. This nucleoside triphosphate hydrolase (NTPDase) has been shown to be secreted into the vacuolar lumen and incorporated into the inner leaflet of the PVM, where CD73 would be localized if it is indeed retained during formation of the PV. In addition, the PV is known to be in close proximity to mitochondria, and *T. gondii* infection has been shown to rapidly deplete the host ATP pool. Thus, one possibility is that adenosine is generated in the PV via the sequential dephosphorylation of host ATP to AMP by the parasite-encoded NTPDase , followed by AMP dephosphorylation by CD73, and culminating in the uptake of adenosine by the transporter on the parasite plasma membrane (Fig 4.1). The major drawback to this model is that it does not account for why the absence of CD73 does not impede tachyzoites replication, which would be predicted if CD73-generated adenosine is necessary for *T. gondii* purine salvage. It is possible that tachyzoites would have additional mechanisms to salvage purines, such as through manipulation of the host cell purine *de novo* pathway.

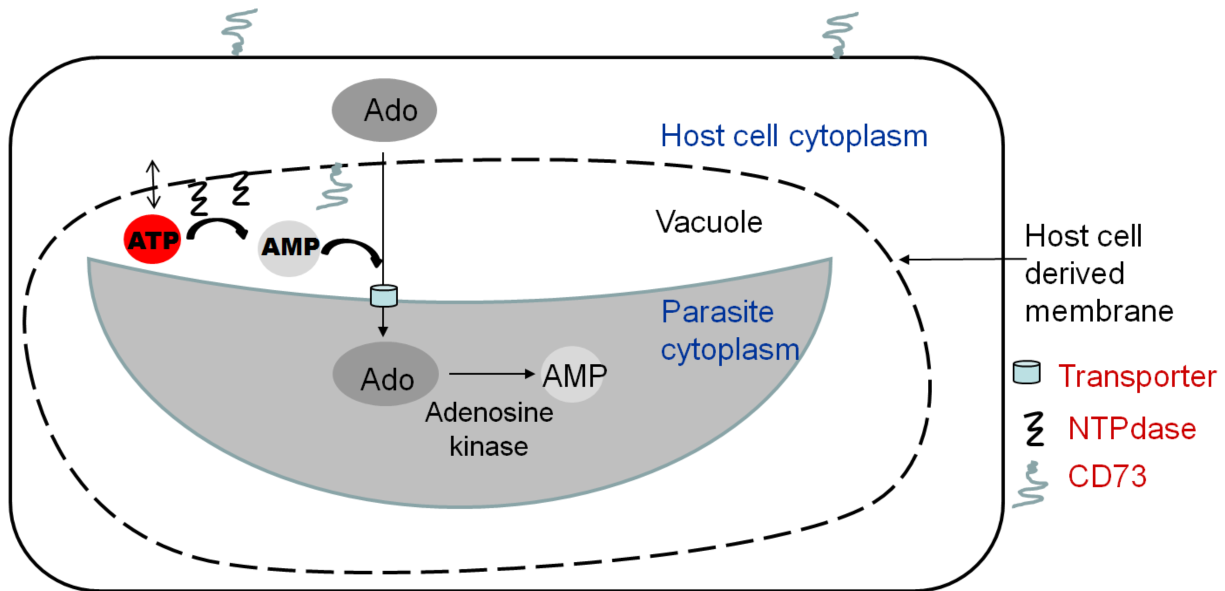


Figure 4.1. Proposed model of how CD73 may contribute to *T. gondii* purine salvage in an infected cell. CD73 is incorporated in the inner leaflet of the PV membrane, where it is situated to generate adenosine from ATP in concert with *T. gondii* NTPDase.

To further investigate the reason for the reduced parasite burden in the CNS, we inoculated WT and CD73$^{-/-}$ mice with *T. gondii* via peritoneal injection. Unexpectedly, CD73$^{-/-}$ mice were markedly susceptible to *T. gondii* intraperitoneal infection. The potential interactions and causes of these seemingly disparate results will be discussed later in this chapter.

In contrast to our findings with oral infection, CD73-/- mice infected with *T. gondii* by the intraperitoneal route were highly susceptible during the acute stage. Infection with a dose of *T. gondii* ME49 cysts that was not lethal for C57BL/6 mice led to a 50-100% mortality rate in CD73$^{-/-}$ mice. The mice began to exhibit increased morbidity within 3 days of infection, suggesting either parasite- or innate immune response-mediated pathology.

To define the mechanism by which CD73 protected mice against systemic *T. gondii* induced inflammation, we infected CD73-/- mice and treated them with the adenosine receptor

agonist NECA. NECA treatment protected CD73-/- mice, suggesting a role for adenosine receptor signaling. To confirm, we infected A_1 and A_{2A} receptor knockout mice, and found that $A_{2A}AR^{-/-}$ mice were as susceptible as $CD73^{-/-}$ mice. Further *in vivo* experiments with bone marrow chimeras and $RAG1^{-/-}$ mice established a role for both hematopoietic and nonhematopoietic lineage cells in generating adenosine via CD73, although T and B cells were not a critical source of CD73-generated adenosine.

Although parasite dissemination to mesenteric lymph nodes was slightly elevated in $CD73^{-/-}$ mice, the greatest differences we saw was in increased production of proinflammatory cytokines IL1β and TNFα, as well as IFNγ at later stages of infection. Our results also showed that nitric oxide production by PECs isolated from infected $CD73^{-/-}$ mice was significantly elevated. Nitric oxide is important for the control of parasite replication, but can also lead to tissue damage and pathology. It would be informative to test whether pharmacological blockade of nitric oxide production protects $CD73^{-/-}$ mice from *T. gondii* induced immunopathology.

A deficit in CD73 enzymatic activity could lead not only to adenosine depletion, but also upstream ATP accumulation. ATP is known to activate the NLRP3 inflammasome via P2X receptor signaling, leading to the release of active IL1β via caspase-1 processing of pro-IL1β (4). It is possible that in CD73-deficient mice, buildup of ATP could lead to inflammasome activation either via P2X7 signaling, or through lack of antagonism by adenosine, which could explain the observed increase in IL1β. However, the results with the A_{2A} receptor knockout mice, which appear to recapitulate the susceptibility observed in $CD73^{-/-}$, suggests a dominant role for adenosine receptor signaling, at least during the early stage of infection. It would be interesting to determine the IL1β levels in A_{2A} $AR^{-/-}$ mice and compare them to CD73-knockout mice. Another approach would be to compare the susceptibility of $CD39^{-/-}$ mice to *T. gondii* ip

infection, although those mice would also have reduced adenosine levels in addition to the expected accumulation of extracellular ATP.

It is interesting that the route of infection with *T. gondii* has a striking effect on the outcome of infection in CD73$^{-/-}$ mice. These mice are resistant to the acute phase of oral infection, yet are highly susceptible to intraperitoneal infection. Similar effects may be seen when comparing different strains of mice, suggesting host genetic factors could impact the outcome of infection (5, 6). For example, Balb/c mice are resistant to both oral and intraperitoneal infection, while C57BL/6 mice are highly susceptible to oral infection but relatively resistant to intraperitoneal infection. And even within the same genetic background, the route of infection can give rise to different responses, as the local immune system may differ in cell composition, tendency to polarize the response towards a Th1, or Th2 or even a regulatory phenotype. Johnson reported a strain resistant to oral *T. gondii* infection while being susceptible to ip infection. It is likely that the regulation of the immune response in the peritoneal cavity requires the presence of adenosine to limit pathology, while other compartments (the GI tract or the CNS during chronic infection) may have multiple or redundant mechanisms to control immunopathology. Notably, the blood-brain barrier prevents neutrophils from accumulating in the CNS, whereas the peritoneal cavity does not have that additional barrier to leukocyte hyper-infiltration.

Aside from reducing pro-inflammatory cascades, extracellular adenosine is also implicated in promoting anti-inflammatory factors such as IL-10 and induction of suppressor T cells. In particular A_{2B} and A_{2A} signaling induces IL-10 release from macrophages and microglia (7-9). Although we saw a modest decrease in IL-10 release from infected PECs from CD73$^{-/-}$ mice, the difference was not statistically significant. It is possible that other regulatory factors,

such as IL-4 and TGFβ, contribute to CD73-mediated protective effects of extracellular adenosine in *T. gondii* ip infection.

In addition to iNOS-generated nitric oxide, interferon gamma induces other protective factors to limit *T. gondii* replication. In infected mouse cells, treatment with IFNγ induces the accumulation of IRG proteins (IFN-inducible GTPases) around the parasitophorous vacuole, leading to membrane disruption and parasite-killing (10, 11). In human cells, The enzyme indoleamine 2,3-dioxygenase (IDO) degrades L-tryptophan, which has been shown to suppress *T. gondii* proliferation in various cells, including macrophages, astrocytes, epithelial cells and fibroblasts (12-14). IDO activity in the CNS is thought to be of greater importance than nitric oxide production by IFNγ-induced iNOS in human host cells. Thus it would be interesting to determine the role of adenosine receptor signaling in IDO-mediated control of *T. gondii* infection in human macrophages or fibroblasts. Yet another pathway that may intersect with the extracellular adenosine-*T. gondii* interaction is the production of reactive oxygen species (ROS). ROS production can be induced by extracellular ATP in *T. gondii* infected macrophages (15), and since it is plausible that ATP or its metabolites, ADP andAMP are elevated in $CD73^{-/-}$ mice, it would be informative to test the contribution of this pathway to the control of *T. gondii* cyst formation in CD73-deficient hosts.

A major surprising result of the acute infection experiments was the notable reduction in IL-12 in $CD73^{-/-}$ mice infected ip with *T. gondii*, despite significantly elevated IFNγ. Both induction of IFNγ and resistance to *T. gondii* infection is established to require IL-12 (16) although limited IFNγ may be produced in the absence of IL-12 (17). One possible cytokine that may be involved is the Th1 cytokine IL-18, which has been shown to be negatively regulated by

adenosine (18), to induce robust production of IL1β and TNFα(19, 20), and to promote IFNγ production independent of IL-12(21).

Based on the ubiquitous expression of CD73 in the brain and the important role of extracellular adenosine in modulating the immune response and maintaining functional barrier integrity in the GI tract, the blood brain barrier, and other important intersections of the immune response, the external environment and the first lines of defense against dangerous pathogens, CD73 and extracellular adenosine present potential targets for therapeutic interventions in many ways.

REFERENCES

1. Bynoe MS, Waickman, A., Mahamed, D.A., Mueller, C., Mills, J.H., Czopik, A. (2012) CD73 Is Critical for the Resolution of Murine Colonic Inflammation. *Journal of Biomedicine and Biotechnology* 2012:13.
2. Kolachala VL, Obertone TS, Wang L, Merlin D, & Sitaraman SV (2006) Adenosine 2b receptor (A2bR) signals through adenylate cyclase (AC) 6 isoform in the intestinal epithelial cells. *Biochim Biophys Acta* 1760(7):1102-1108.
3. Mordue DG, Desai N, Dustin M, & Sibley LD (1999) Invasion by Toxoplasma gondii establishes a moving junction that selectively excludes host cell plasma membrane proteins on the basis of their membrane anchoring. *J Exp Med* 190(12):1783-1792.
4. Solle M*, et al.* (2001) Altered cytokine production in mice lacking P2X(7) receptors. *J Biol Chem* 276(1):125-132.
5. Johnson AM (1984) Strain-dependent, route of challenge-dependent, murine susceptibility to toxoplasmosis. *Z Parasitenkd* 70(3):303-309.
6. McLeod R*, et al.* (1989) Immune responses associated with early survival after peroral infection with Toxoplasma gondii. *Journal of immunology* 142(9):3247-3255.
7. Le Moine O*, et al.* (1996) Adenosine enhances IL-10 secretion by human monocytes. *J Immunol* 156(11):4408-4414.
8. Koscso B*, et al.* (2012) Adenosine augments IL-10 production by microglial cells through an A2B adenosine receptor-mediated process. *J Immunol* 188(1):445-453.
9. Hasko G*, et al.* (1996) Adenosine receptor agonists differentially regulate IL-10, TNF-alpha, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice. *J Immunol* 157(10):4634-4640.

10. Taylor GA, Feng CG, & Sher A (2004) p47 GTPases: regulators of immunity to intracellular pathogens. *Nat Rev Immunol* 4(2):100-109.

11. Butcher BA*, et al.* (2005) p47 GTPases regulate Toxoplasma gondii survival in activated macrophages. *Infect Immun* 73(6):3278-3286.

12. Murray HW*, et al.* (1989) Role of tryptophan degradation in respiratory burst-independent antimicrobial activity of gamma interferon-stimulated human macrophages. *Infect Immun* 57(3):845-849.

13. Daubener W*, et al.* (1996) Anti-parasitic effector mechanisms in human brain tumor cells: role of interferon-gamma and tumor necrosis factor-alpha. *Eur J Immunol* 26(2):487-492.

14. Oberdorfer C, Adams O, MacKenzie CR, De Groot CJ, & Daubener W (2003) Role of IDO activation in anti-microbial defense in human native astrocytes. *Adv Exp Med Biol* 527:15-26.

15. Correa G, Marques da Silva C, de Abreu Moreira-Souza AC, Vommaro RC, & Coutinho-Silva R (2010) Activation of the P2X(7) receptor triggers the elimination of Toxoplasma gondii tachyzoites from infected macrophages. *Microbes Infect* 12(6):497-504.

16. Lieberman LA*, et al.* (2004) IL-23 provides a limited mechanism of resistance to acute toxoplasmosis in the absence of IL-12. *J Immunol* 173(3):1887-1893.

17. Scharton-Kersten T, Caspar P, Sher A, & Denkers EY (1996) Toxoplasma gondii: evidence for interleukin-12-dependent and-independent pathways of interferon-gamma production induced by an attenuated parasite strain. *Exp Parasitol* 84(2):102-114.

18. Takahashi HK*, et al.* (2007) Adenosine A2A-receptor stimulation inhibits lipopolysaccharide-induced interleukin-18 production in monocytes. *J Pharmacol Sci* 104(2):183-186.

19. Netea MG, Kullberg BJ, Verschueren I, & Van Der Meer JW (2000) Interleukin-18 induces production of proinflammatory cytokines in mice: no intermediate role for the cytokines of the tumor necrosis factor family and interleukin-1beta. *Eur J Immunol* 30(10):3057-3060.

20. Joosten LA*, et al.* (2004) Interleukin-18 promotes joint inflammation and induces interleukin-1-driven cartilage destruction. *Am J Pathol* 165(3):959-967.

21. Xing Z, Zganiacz A, Wang J, Divangahi M, & Nawaz F (2000) IL-12-independent Th1-type immune responses to respiratory viral infection: requirement of IL-18 for IFN-gamma release in the lung but not for the differentiation of viral-reactive Th1-type lymphocytes. *J Immunol* 164(5):2575-2584.

APPENDIX A

Table 1. Murine and *T. gondii* primer sequences used in real-time PCR.

Gene [a]	Forward Primer	Reverse Primer
PGK1	CTCCGCTTTCATGTAGAGGAAG	GACATCTCCTAGTTTGGACAGTG
CD73	CAAATCCCACACAACCACTG	TGCTCACTTGGTCACAGGAC
IFNγ	CTTTAACAGCAGGCCAGACA	GCGAGTTATTTGTCATTCGG
iNOS	ACATCGACCCGTCCACAGTAT	CAGAGGGGTAGGCTTGTCTC
TNFα	GGTATGAGATAGCAAATCGGC	CAGACCCTCACACTCAGATC
MadCAM1	GGCAGCAGTATCCTCTCTGTAC	GCATGGTGACCTGGCAGTGAAG
VCAM1	TGCCGAGCTAAATTACACATTG	CCTTGTGGAGGGATGTACAGA
CX3CL1	GTGCTGACCCGAAGGAGAAA	CACCCGCTTCTCAAACTTGC
T. gondii Tubulin 1	CGCCACGGCCGCTACCTGACT	TACGCGCCTTCCTCTGCACCC
T. gondii SAG1	ATCGCCTGAGAAGCATCACTG	CGAAAATGGAAACGTGACTGG
T. gondii BAG1	GACGTGGAGTTCGACAGCAAA	ATGGCTCCGTTGTCGACTTCT
T. gondii B1 gene	GGAGGACTGGCAACCTGGTGTCG	TTGTTTCACCCGGACCGTTTAGCAG

[a]PGK1, phosphoglycerate kinase 1; IFNγ, interferon gamma; iNOS, inducible nitric oxide synthase; TNFα, tumor necrosis factor alpha; MadCAM1, mucosal vascular addressin and cell adhesion molecule 1; VCAM1, vascular cell adhesion molecule 1; CX3CL1, chemokine (C-X3-C motif) ligand 1

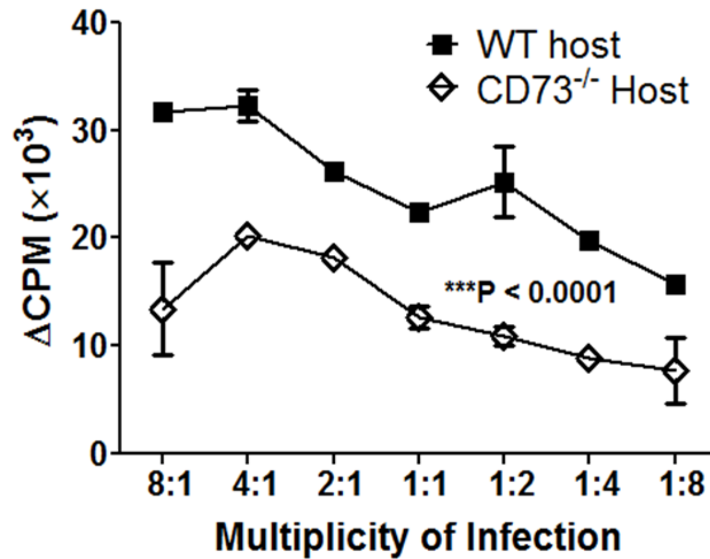
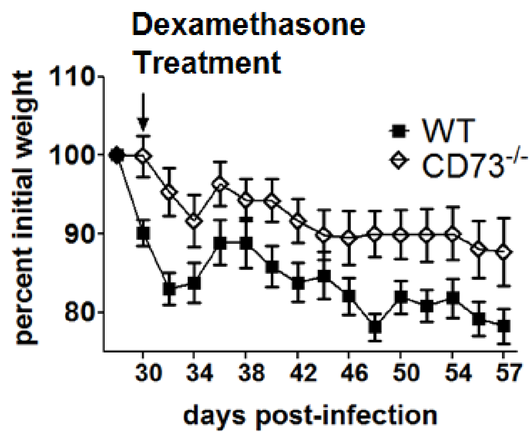


Figure. A1. . Proliferation of T. gondii bradyzoites isolated from wildtype and $CD73^{-/-}$ hosts. C57BL/6 and $CD73^{-/-}$ mice were infected with 10 ME49 cysts and brain cysts isolated 8 weeks later. Bradyzoites were liberated and inoculated into human fibroblasts at various multiplicities of infection. Incorporation of tritiated uracil was used to determine proliferation. Error bars represent the s.e.m.



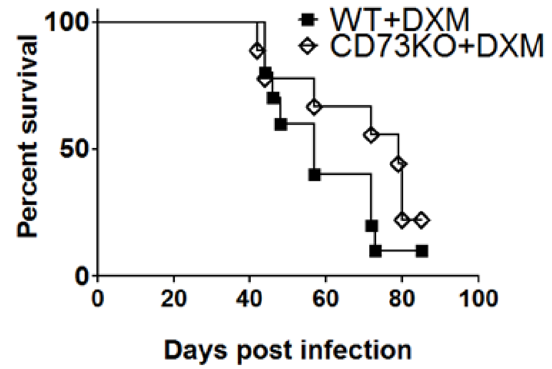


Figure A2. Dexamethasone induced recrudescence of chronic *T. gondii* infection. Wildtype and CD73$^{-/-}$ were orally infected with 10 ME49 cysts. Thirty days later, mice were treated with dexamethasone in the drinking water (arrow) and monitored for weight loss, mortality and morbidity. (A) Weight loss (B) Survival.