BORRELLIDIN, A THREONYL-TRNA SYNTHETASE INHIBITOR, IS A POTENT IMMUNOSUPPRESSIVE AND ANTI-INFLAMMATORY AGENT

by

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(Experimental Medicine)

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(Vancouver)

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Abstract

Excessive or dysregulated immune responses require clinical intervention to prevent tissue damage and organ dysfunction. Unfortunately, many of these clinical interventions have undesired side effects; therefore, development of novel therapeutic agents with different mechanisms of action would be immensely beneficial. Mounting evidence in *in vitro* and *in vivo* studies implicate amino acid deprivation (AAD) as a key natural mechanism by which the body regulates immune responses and suppresses immune cell activation and function. However, the role of aminoacyl-tRNA synthetase (aaRS) inhibitors in regulating immune responses is largely unknown. Since inhibitors of aaRSs limit the cell’s availability to specific amino acids and thereby creating an amino acid limiting environment, a deeper investigation of aaRS inhibitor-induced amino acid deprivation and its ability to regulate immune cell function is needed. We hypothesized that aminoacyl tRNA synthetase inhibitors might represent a novel class of immunosuppressive and/or anti-inflammatory agents that act as pharmacomimetics of amino acid deprivation.

Two specific aims were accomplished in this study. We first showed that borrelidin is a potent inhibitor of T-cell proliferation, activation and cytokine production. As compared with other primary cells and cell lines, we determined borrelidin is most effective at suppressing T-cells. We then showed borrelidin potently suppresses lipopolysaccharide (LPS) induced- release of inflammatory cytokines such as TNF alpha (TNF\(\alpha\)) from primary splenocytes and suppression of TNF\(\alpha\) occurs at the level of protein synthesis. In both T-cells and macrophages, intracellular staining and flow cytometry identified that borrelidin promotes activation of the general control non-derepressible 2 (GCN2) stress response pathway and inhibition of the mammalian target of rapamycin (mTOR) pathway.
The findings presented in this thesis collectively demonstrate that borrelidin is a potent immunosuppressive and anti-inflammatory agent. These findings help us to better understand the role of aminoacyl-tRNA synthetase inhibitors in regulating immune function.
Preface

The work presented in this thesis was performed at the Vancouver Prostate Centre under the guidance and support of Dr. Christopher Ong. Nadya Ogloff was responsible for the design, conducting research, data analysis, and preparation of this manuscript. The work described in this thesis has been conducted with the approval of the University of British Columbia Animal Care Committee and Office of Research Ethics under the certificate numbers: A09-0397 and H05-70537.
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<thead>
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>4E-BP1</td>
<td>eIF4e binding protein 1</td>
</tr>
<tr>
<td>AAD</td>
<td>amino acid deprivation</td>
</tr>
<tr>
<td>aaRS</td>
<td>aminoacyl-tRNA synthetase</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>APC</td>
<td>allophycocyanin</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ATF4</td>
<td>activating transcription factor 4</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BMMMC</td>
<td>bone marrow derived mast cells</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD3ζ</td>
<td>T-cell antigen receptor ζ</td>
</tr>
<tr>
<td>CHOP</td>
<td>CCAAT/enhancer-binding protein homologous protein</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>eIF2α</td>
<td>eukaryotic initiation factor 2 alpha</td>
</tr>
<tr>
<td>eIF4E</td>
<td>eukaryotic translation initiation factor 4E</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbant assay</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>FOXP3</td>
<td>forkhead box P3</td>
</tr>
<tr>
<td>GCN2</td>
<td>general control non-derepressed 2</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>GSK3</td>
<td>glycogen synthase kinase 3</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>3-HAA</td>
<td>3-hydroxyanthranillic acid</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalamic-pituitary-adrenal axis</td>
</tr>
<tr>
<td>IC₅₀</td>
<td>half maximal inhibitory concentration</td>
</tr>
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<td>IDO</td>
<td>indoleamine-2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<tr>
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<td>interleukin</td>
</tr>
<tr>
<td>IL4I1</td>
<td>IL-4 induced gene 1</td>
</tr>
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<td>ISR</td>
<td>integrated stress response</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>mCSF-1</td>
<td>murine colony stimulating factor 1</td>
</tr>
<tr>
<td>MDSC</td>
<td>myeloid-derived suppressor cell</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>MgCl</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>MIP</td>
<td>macrophage inflammatory protein</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>---------</td>
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</tr>
<tr>
<td>MLR</td>
<td>mixed leukocyte reaction</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>1-MT</td>
<td>1-methyl-tryptophan</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
</tr>
<tr>
<td>mTORC1</td>
<td>mTOR complex 1</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NALA</td>
<td>N-acetyl-leucine-amide</td>
</tr>
<tr>
<td>NGS</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>ammonium chloride</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory</td>
</tr>
<tr>
<td>OTA</td>
<td>ochratoxin A</td>
</tr>
<tr>
<td>p70⁵⁶k</td>
<td>70kDa ribosomal protein S6 kinase</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>PheRS</td>
<td>phenylalanyl-tRNA synthetase</td>
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<tr>
<td>PI3K</td>
<td>phosphoinositol 3-kinase</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PPi</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPL32</td>
<td>60S ribosomal protein L32</td>
</tr>
<tr>
<td>RPM</td>
<td>rotations per minute</td>
</tr>
<tr>
<td>rpS6</td>
<td>ribosomal protein S6</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>real time PCR</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TBST</td>
<td>tris-buffered saline – Tween-20</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TH</td>
<td>T-helper cell</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>ThrRS</td>
<td>threonine-tRNA synthetase</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>TPBS</td>
<td>tris-phosphate buffered saline</td>
</tr>
<tr>
<td>Treg</td>
<td>T-regulatory cell</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>tris hydrochloric acid</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer RNA</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>TSC</td>
<td>tuberous sclerosis complex</td>
</tr>
</tbody>
</table>
Acknowledgements

First and foremost, I would like to express my deepest gratitude to my supervisor, Dr. Christopher Ong for his continuous support, guidance, and patience during the course of my studies. He was an exceptional supervisor, mentor, and friend.

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CHAPTER 1. Introduction

1.1 Introduction

1.1.1 The immune system and its regulation

Humans are exposed to millions of potential pathogens daily; through contact, ingestion, and inhalation. The ability to avoid infection depends, in part, on the adaptive immune system. Composed of highly specialized, systemic cells, the adaptive immune system remembers previous encounters with specific pathogens and efficiently attacks and destroys them when they appear subsequent times [1,2]. However, the adaptive immune system is slow to develop, taking approximately one week for specific clones of B and T lymphocytes to become activated, expand, and respond to the threat [3]. Fortunately, an evolutionarily older first line of defence exists to protect against exposure to new pathogens in a non-specific manner. The innate immune system recognizes conserved patterns on pathogens to initiate an inflammatory response against invading pathogens and help activate and direct the adaptive immune response [4-6]. The innate and adaptive immune systems function together to effectively protect against pathogens and foreign substances.

Unfortunately, one caveat of the immune system is that excessive or uncontrolled immune responses can lead to a diverse array of immunological diseases including asthma, allergies, rheumatoid arthritis, inflammatory bowel disease, septic shock, atherosclerosis, and many others. Unwelcome immune responses are also mounted against a new organ or tissue during transplantation, since the immune system views the new organ or tissue as foreign. As such, these unwanted immune responses require clinical intervention to prevent tissue damage and organ dysfunction.
1.1.2 Current immunosuppressive and anti-inflammatory therapies

Development of immunosuppressive and anti-inflammatory therapies and regimes has had a beneficial effect on morbidity and mortality in transplantation and immune-related diseases. These current therapies focus on blocking the activity or efficacy of the immune system and are often given in combination being that many of these agents act synergistically (Table 1.1). Indeed, the introduction of immunosuppressive and anti-inflammatory therapies greatly improved survival rates for organ transplantation [7,8] and decreased the severity of a number of immune-related diseases [9-13].

Table 1.1 Immunosuppressive and anti-inflammatory drugs and their side effects

<table>
<thead>
<tr>
<th>Class</th>
<th>Drug</th>
<th>Mode of Action</th>
<th>Side Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunosuppressive Drugs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>Methylprednisolone</td>
<td>A class of steroid hormones that bind to the glucocorticoid receptor and mediate pleiotropic responses including anti-inflammatory effects</td>
<td>Fluid retention, weight gain, diabetes, bone mineral loss, dyslipidemia, irritability</td>
</tr>
<tr>
<td>Anti-proliferative</td>
<td>Azathioprine</td>
<td>Interferes with nucleotide synthesis and results in inhibition of T-cell activation</td>
<td>Bone marrow suppression, hepatotoxicity, acute pancreatitis[14]</td>
</tr>
<tr>
<td></td>
<td>Cyclophosphamide</td>
<td>Interferes with DNA synthesis</td>
<td>Myelosuppression, vomiting, nausea, gastrointestinal discomfort, hemorrhagic cystitis and hair loss</td>
</tr>
<tr>
<td></td>
<td>Mycophenolate mofetil (MMF)</td>
<td>Selective inhibitor of <em>de novo</em> pathway of purine biosynthesis. Inhibits T and B-cell proliferation</td>
<td>Diarrhea, anemia, leucopenia, may increase cytomegalovirus disease, abdominal pain [15]</td>
</tr>
<tr>
<td>Calcineurin inhibitors</td>
<td>Cyclosporin A, Tacrolimus</td>
<td>Block production of IL-2 and inhibit T-cell proliferation and differentiation</td>
<td>Nephrotoxicity, hypertension, hyperlipidemia, hirsutism, gingival hyperplasia, tremors, post-transplant malignancies[16]</td>
</tr>
<tr>
<td>Target of rapamycin (TOR) inhibitors</td>
<td>Sirolimus, Everolimus</td>
<td>Prevent activation of mTOR; prevents cytokine receptors from activating cell cycle</td>
<td>Hyperlipidemia, thrombocytopenia, diarrhea, poor wound healing</td>
</tr>
<tr>
<td>Class</td>
<td>Drug</td>
<td>Mode of Action</td>
<td>Side Effects</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------------------------------------</td>
<td>-----------------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Depleting antibodies</td>
<td>Antithymocyte globulin (ATG)</td>
<td>Depletes all lymphoid cells</td>
<td>Lymphopenia, increased risk of infection, thrombocytopenia, cytokine release syndrome, serum sickness</td>
</tr>
<tr>
<td></td>
<td>Anti-CD3 mAb</td>
<td>Depletes CD3+ T-cells</td>
<td>Cytokine release syndrome</td>
</tr>
<tr>
<td></td>
<td>Alemtuzumab</td>
<td>mAb against CD52, Depletes B-cells, T-cells NK cells and monocytes</td>
<td>Neutropenia, anemia, autoimmunity</td>
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<td></td>
<td>Rituximab</td>
<td>mAb against CD20, Depletes most B cells</td>
<td>Some infusion related reactions. Possible reactivation of hepatitis B. Impaired B cell function</td>
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<tr>
<td>Non-depleting antibodies or proteins</td>
<td>Daclizumab, Basiliximab</td>
<td>mAb against CD25, Binds to activated T-cells</td>
<td>Hypersensitivity reactions, abdominal discomfort and myalgia</td>
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<tr>
<td></td>
<td>CTLA4-Ig</td>
<td>Blocks co-stimulation of T-cells</td>
<td>Increased risk of thromboembolism and upper respiratory tract infection</td>
</tr>
<tr>
<td></td>
<td>Intravenous immunoglobulin</td>
<td>Pooled human plasma from thousands of volunteers. Modulation of Fc receptors, interferences with activity of complement, cytokine and idioype network. Regulation of cell growth and effects on activation and function of dendritic cells, T-cells and B-cells</td>
<td>Acute renal toxicity, hypersensitivity reactions, hemolytic anemia, urticarial rashes, hemolysis, cytopenia</td>
</tr>
<tr>
<td>Anti-Inflammatory</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steroids/ Glucocorticoids</td>
<td>Naturally occurring glucocorticoids (ie. Cortisol)</td>
<td>Non-specific anti-inflammatory. Repression of gene transcription</td>
<td>Increased risk of infection, depression, hypertension, Muscle atrophy, latent diabetes mellitus, stomach upset, glaucoma[17]</td>
</tr>
<tr>
<td></td>
<td>Synthetic glucocorticoids (ie. Prednisone, Dexamethasone, Methylprednisolone, Betamethasone )</td>
<td>Non-specific anti-inflammatory. Repression of gene transcription</td>
<td>Increased risk of infection, depression, hypertension, Muscle atrophy, latent diabetes mellitus, stomach upset, glaucoma[17]</td>
</tr>
<tr>
<td>Class</td>
<td>Drug</td>
<td>Mode of Action</td>
<td>Side Effects</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>-------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------------------</td>
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<tr>
<td>Nonsteroidal anti-inflammatory drugs (NSAID)</td>
<td>Salicylates (ie acetylsalicylic acid)</td>
<td>inhibit the production of prostaglandins by blocking the COX enzyme by noncompetitive and irreversible acetylation</td>
<td>Increased risk of gastrointestinal bleeding [18], dyspepsia [19], hives, swelling, headache, hyperkalemia, increased risk of myocardial infarction, acute renal failure, hypertension[20]</td>
</tr>
<tr>
<td></td>
<td>p-amino phenol derivatives (ie. Paracetamol)</td>
<td>inhibit the production of prostaglandins by competitively and reversibly inhibiting the COX enzyme</td>
<td>Increased risk of gastrointestinal bleeding [18], dyspepsia [19], hives, swelling, headache, hyperkalemia, increased risk of myocardial infarction, acute renal failure, hypertension[20]</td>
</tr>
<tr>
<td></td>
<td>Propionic acid derivatives (ie. Ibuprofen)</td>
<td>inhibit the production of prostaglandins by competitively and reversibly inhibiting the COX enzyme</td>
<td>Increased risk of gastrointestinal bleeding [18], dyspepsia [19], hives, swelling, headache, hyperkalemia, increased risk of myocardial infarction, acute renal failure, hypertension[20]</td>
</tr>
<tr>
<td></td>
<td>Acetic acid derivatives (ie. Indomethacin)</td>
<td>inhibit the production of prostaglandins by competitively and reversibly inhibiting the COX enzyme</td>
<td>Increased risk of gastrointestinal bleeding [18], dyspepsia [19], hives, swelling, headache, hyperkalemia, increased risk of myocardial infarction, acute renal failure, hypertension[20]</td>
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<tr>
<td></td>
<td>Enolic acid (Oxicam) derivatives (ie. Piroxicam)</td>
<td>inhibit the production of prostaglandins by competitively and reversibly inhibiting the COX enzyme</td>
<td>Increased risk of gastrointestinal bleeding [18], dyspepsia [19], hives, swelling, headache, hyperkalemia, increased risk of myocardial infarction, acute renal failure, hypertension[20]</td>
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<td></td>
<td>Fenamic acid derivatives (Fenamates) (ie. Mefenamic acid)</td>
<td>inhibit the production of prostaglandins by competitively and reversibly inhibiting the COX enzyme</td>
<td>Increased risk of gastrointestinal bleeding [18], dyspepsia [19], hives, swelling, headache, hyperkalemia, increased risk of myocardial infarction, acute renal failure, hypertension[20]</td>
</tr>
<tr>
<td></td>
<td>Selective COX-2 inhibitors (Coxibs) (ie. Celecoxib)</td>
<td>inhibit the production of prostaglandins by competitively and reversibly inhibiting the COX enzyme</td>
<td>Increased risk of gastrointestinal bleeding [18], dyspepsia [19], hives, swelling, headache, hyperkalemia, increased risk of myocardial infarction, acute renal failure, hypertension[20]</td>
</tr>
<tr>
<td>Class</td>
<td>Drug</td>
<td>Mode of Action</td>
<td>Side Effects</td>
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</tr>
<tr>
<td>Sulphonanilides (ie. Nimesulide)</td>
<td>inhibit the production of prostaglandins by competitively and reversibly inhibiting the COX enzyme</td>
<td>Increased risk of gastrointestinal bleeding [18], dyspepsia [19], hives, swelling, headache, hyperkalemia, increased risk of myocardial infarction, acute renal failure, hypertension[20]</td>
<td></td>
</tr>
<tr>
<td>Immune Selective Anti-Inflammatory Derivatives (ImSAIDs)</td>
<td>Inhibit excessive activation and migration of inflammatory cells</td>
<td>N/A not tested in humans yet</td>
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mAb: monoclonal antibody  Ig: immunoglobulin; immunosuppressive drugs adapted from [21] unless otherwise referenced.

Since the discovery of glucocorticoids in the 1940s and the recognition of their anti-inflammatory effects, they have been amongst the most widely used and effective treatments to control chronic inflammatory and immune-related diseases; however, clinical efficacy of glucocorticoids is compromised by the side effects of long-term treatment. Among others, these side effects include cardiovascular disease [22,23], insulin resistance/type2 diabetes mellitus [24], and osteoporosis [25] (Table 1.1). The underlying precise molecular mechanisms of the side effects are still unknown or there are several mechanisms that seem to be involved. In some cases, the side effects are mediated predominantly by transrepression (e.g. suppression of the hypothalamic-pituitary-adrenal (HPA) axis [26-28]) whereas others are predominantly mediated by transactivation (e.g. diabetes [29], glaucoma [30]).

Despite the intense efforts by the pharmaceutical industry, it has proven extraordinarily difficult to separate the therapeutic, anti-inflammatory and immunosuppressive activities of glucocorticoids from their adverse side effects or to find any new treatments that come close to the therapeutic benefits of glucocorticoids. As a whole, the side effects of glucocorticoids are the limiting factor for the use of these agents in the treatment of a variety of diseases. There is, therefore, currently a large unmet medical need for development of new and more potent...
immune-modulating agents with unique or novel mechanisms of action that show high synergistic efficiency with current agents in order to minimize any associated toxicities. Development of new agents may lead to a higher rate of graft tolerability with fewer side effects, facilitate long-term graft survival, and improve patient quality of life and survival.

1.2 Natural immunosuppression through amino acid deprivation

In an effort to identify new anti-inflammatory and immunosuppressive drugs, we looked to nature for inspiration. One key natural mechanisms by which the body regulates inflammation and immunosuppression is through localized depletion of amino acids by amino acid catabolizing enzymes, such as indoleamine-2,3-dioxygenase (IDO) [31,32].

1.2.1 Indoleamine-2,3-dioxygenase (IDO) as a local immunosuppressive factor

The best characterized example of immunosuppression by amino acid depletion is tryptophan catabolism through the kynurenine pathway. IDO is an enzyme that catalyzes the initial and rate-limiting step in this pathway by converting the essential amino acid, L-tryptophan, into kynurenine; leading to the biosynthesis of nicotinamide adenine dinucleotide (NAD) [32]. IDO has an high affinity for L-tryptophan (Km = 0.02 mM) and can rapidly catabolize it to create a localized microenvironment devoid of this essential amino acid [33]. L-tryptophan is required for protein synthesis: it cannot be synthesized de novo and therefore must be acquired by humans from their diet [34]. Of the dietary tryptophan that is not used in protein synthesis, 99% is metabolized along the kynurenine pathway by IDO [35].
IDO is intracellularly expressed in a constitutive or inducible manner in many different cells and tissues. In macrophages [36], dendritic cells [37,38], placenta [39], and cancer cell lines [40,41] the cytokine interferon-gamma (IFN-γ) is a potent inducer of IDO expression. The immunomodulatory and anti-proliferative effects of IFN-γ, both *in vitro* and *in vivo*, seem to be in, part due, to IFN-γ inducing cellular proteins such as IDO. Tumor necrosis factor α (TNFα), interleukin-10 (IL-10), and toll-like receptor (TLR) activation has also been reported to induce IDO gene transcription and activity [42-45]. IDO has also been detected in cells types important in allergic inflammation including eosinophils [46], endothelial cells [47], and lung epithelial cells [48]. The lower gastrointestinal (GI) tract is the only known location that IDO is expressed constitutively — this is due to the presence of bacteria; for example, gnotobiotic mice do not constitutively express IDO in the lower GI tract [49]. The ability to regulate expression levels of IDO in response to external stimuli is logical given the plasticity of antigen presenting cells (APCs) — sometimes APCs present antigens in an activating fashion, and other times in a tolerizing fashion, depending on the context and other signals involved [50].

The initial role of IDO was thought to be largely antimicrobial by reducing the availability tryptophan to microorganisms in an inflammatory microenvironment. In more recent years, IDO has been shown to have a profound immunoregulatory activity. An example of the role IDO plays in regulating the immune response is found in the ability of the fetus to suppress the maternal immune system during pregnancy [51,52]. Trophoblast cells of the placenta express high levels of IDO that creates a zone of low tryptophan levels surrounding the fetus; so when T-cells and immune cells enter this low tryptophan containing microenvironment they become anergic and unresponsive [51]. This protection is thought to be IDO-dependent, as treatment of pregnant mice with the IDO inhibitor, 1-methyl-tryptophan (1-MT), results in T-cell mediated rejection of their semi-allogeneic fetus [51]. These findings suggest IDO expression in
trophoblasts creates an immunosuppressive barrier to protect the semi-allogeneic fetus from maternal immune system.

IDO has subsequently been found to play an important role in the regulation of many different types of immune responses: protecting against autoimmunity [53,54]; inflammatory pathologies such as, colitis [55], rheumatoid arthritis [56], and granulomatous diseases [57]; and allergy [58,59]. In addition, IDO contributes to the host’s immune unresponsiveness to neoplasia [60-62] and viral infection [63]. The wide spectrum of physiopathological conditions in which IDO is at work suggests that multiple mechanisms are used by their effector systems to downregulate immune and inflammatory responses.

1.2.2 Other amino acid catabolizing enzymes as immunosuppressive factors

In addition to IDO, arginine and phenylalanine metabolizing enzymes have been implicated in influencing an immune response.

L-Arginine plays a central role in several functions of the immune system. It is a semi-essential amino acid that is metabolized in virtually all mammalian cells and tissues by the T helper 1 (T₇₁) cytokine-regulated inducible nitric oxide synthase (NOS) to produce nitric oxide. Arginine can also be metabolized by arginase, to produce L-ornithine and urea. Arginase I is constitutively expressed in granulocytes and in the liver and can be induced in macrophages upon T₇₂ cytokine exposure [64-66]. A second isoform of arginase, arginase II, also exists and is widely expressed at low levels in the mitochondria of several cells and tissues in the body; however it is not thought to play an immune regulating role [66]. Upregulation of arginase I in murine peritoneal macrophages leads to a rapid reduction in extracellular levels of L-arginine, induces decreased expression of the T-cell antigen receptor ζ (CD3ζ), and diminishes
proliferation in T-cells [67]. This inhibition was reversed and proliferation was recovered by the addition of arginase I competitive inhibitors or by the addition of excess L-arginine. Likewise, NOS expression in alveolar macrophages plays a key role in the downregulation of T-cell activation in the lung [68].

Phenylalanine metabolism is also suggested to have tolerogenic and immunosuppressive activities. IL-4-induced gene 1 (IL4I1) is a secreted L-phenylalanine oxidase that, by oxidative de-amination of phenylalanine, produces hydrogen peroxide (H₂O₂) and phenylpyruvate. IL4I1 expression in dendritic cells and monocytes inhibits the proliferation of CD3-stimulated T-cells via H₂O₂ production and inhibits inflammatory cytokine and chemokine secretion in vitro [69,70]. Therefore, by depriving the local microenvironment of relevant amino acids, amino acid catabolising enzymes regulate T-cell activation and general immune function.

1.2.3 Amino acid levels can directly regulate an immune response

In addition to tryptophan, arginine, and phenylalanine depletion, controlling the presence or abundance of other amino acids, histidine and valine, is emerging as an important strategy of immune regulation. T-cells fail to proliferate in response to antigen when any one, or more, of these amino acids are limiting [71]. As previously discussed, a decrease in L-arginine by amino acid catabolising enzymes, arginase I, yields reduced proliferation and decreased expression of CD3ζ. This response is also seen in Jurkat T-cells when cultured in medium without L-arginine [72].

Another essential amino acid for T-cell proliferation is L-cysteine. T-cells lack cystathionase, an enzyme found in most mammalian cells that is required for conversion of methionine to cysteine. They also lack an intact xC⁻ transporter and therefore cannot import
cystine, a dimeric amino acid formed of two L-cysteine residues that are covalently linked by a disulfide bond, and reduce it intracellularly to L-cysteine [73,74]. Thus, T-cells depend entirely on an external supply of L-cysteine. Dendritic cells promote T-cell activation by taking up cystine, from the environment, reducing it intracellularly to L-cysteine, and then directly providing L-cysteine to T-cells [75]. Myeloid-derived suppressor cells (MDSC) impede this partnership between dendritic cells and T-cells by competing with APCs for extracellular cystine, thereby limiting the pool of cysteine available to T-cell, leading to inhibition of T-cell activation and function [73]. In mice, administration of L-glutamine can regulate gut-starvation induced inflammation and improves IgA-mediated protection against viruses and bacteria in the upper respiratory tract. [76,77].

These findings suggest that depletion of any essential amino acid can impair T-cell proliferation in a similar manner to the well-established depletion of tryptophan by IDO and that adding amino acids back into the environment can recapitulate T-cell function.

### 1.3 Nutrient sensing pathways respond to amino acid deprivation

As described above, there is compelling evidence to confirm amino acid depletion yields a potent immunosuppressive effect on T-cells, both in vitro and in vivo. Recent studies suggest that the stress-responsive kinase general control non-derepressible 2 (GCN2) signaling pathway enables T-cells to sense and respond to the low amino acid conditions generated by IDO [78]. Other studies show reduction in amino acid levels by IDO antagonizes the mammalian target of rapamycin (mTOR) pathway to elicit an immunosuppressive effect [71]. Most likely, both of these nutrient-sensing pathways play a role in allowing the cell to respond to amino acid deprivation (AAD).
1.3.1 GCN2 pathway as a sensor for amino acid deprivation

AAD is able to regulate an immune response, in part, through activating the stress-responsive kinase, GCN2 [78,79]. GCN2 was originally characterized in yeast, where it functions to sense and respond to amino acid starvation. AAD can lead to an increase in the intracellular levels of non-aminoacylated, “uncharged” tRNAs that bind the histidyl-tRNA synthetase homologous domain of GCN2, and induce a conformational change that activates the kinase domain (Figure 1.1); resulting in the phosphorylation of eukaryotic initiation factor 2 (eIF2) on serine 51 of its α subunit [80,81]. eIF2α is a primary and essential component required for protein translation — phosphorylation of eIF2α prevents recycling of eIF2α-GDP to its functional eIF2α-GTP form, leading to decreased global protein translation while simultaneously enhancing translation and expression of selected mRNAs such as activating transcription factor 4 (ATF4) [82]. Elevated levels of ATF4 lead to induction of ATF3 and CHOP (CCAAT/enhancer-binding protein homologous protein)/ GADD153 (growth arrest and DNA-damage-inducible protein153), which together induce gene expression important for cellular remediation and apoptosis [83,84].
Figure 1.1 Mechanism of amino acid deprivation-induced GCN2 pathway activation
AAD causes a rise in the levels of uncharged tRNA, which activates the GCN2 kinase and
initiates downstream signaling. This leads to phosphorylation of eIF2α and ultimately increases
levels of CHOP and other stress-response genes.

The importance of GCN2 as a crucial nutrient sensor is highlighted when studying rats on
indispensable (essential) amino acid (IAA) deficient diets. In rats maintained on IAA deficient
diets, phosphorylation of eIF2α was detected in the anterior of the rat brain piriform cortex, and
inactivation of GCN2 diminished the observed phosphorylated eIF2α levels [85,86].
Furthermore, wild-type rats are able to avoid IAA deficient diets, while GCN2 knockout rats
failed to reject diets poor in IAA. These data suggest the GCN2 pathway recognizes IAA
deficiency in the output neurons of the rat brain piriform cortex, and thus affects the feeding
behaviour of rats by activating neuronal circuit that biases consumption against imbalanced food
sources.

Several experiments corroborated the evidence that the GCN2 pathway is a bridge that
connects AAD with increased immunosuppressive activity. L-tryptophan depletion by IDO-
expressing dendritic cells results in GCN2-dependent down-regulation of CD3ζ in mouse CD8⁺
T-cells, suppression of T-cell proliferation, and induction of a regulatory T-cell phenotype (CD25+, CD69-, CD45RB<sup>low</sup>, CD62L+, CTLA-4+, BTLA<sup>low</sup> and GITR+) capable of controlling diabetogenic T-cells when transferred in vivo [78,79]. Furthermore, activation of GCN2 by amino acid depletion inhibits Th17 differentiation, which can be reversed by adding an excess of free amino acids, supporting the notion that amino acid depletion is sufficient for the repression of Th17 differentiation [87]. Similar immunosuppressive effects were also seen upon L-arginine depletion: stimulated T-cells cultured in the absence of L-arginine show proliferative arrest and an inability to up-regulate cyclin D3, a component of cell cycle machinery that is required for progression of early G1 into S phase of the cell cycle [88]. T-cells from GCN2 knock-out mice did not show this decrease proliferation and were able to up-regulate cyclin D3 suggesting that GCN2 was required for the T-cells to respond to the low L-arginine levels. In contrast, loss of GCN2 enhances immunosuppression by asparaginase indicating GCN2 is broadly required for proper management of stress from amino acid depletion [89].

Cumulatively, these findings suggest that the GCN2 pathway is one downstream sensor of amino acid levels and, upon activation in T-cells, can elicit an immunosuppressive effect.

**1.3.2 Mammalian target of rapamycin as a sensor of amino acid deprivation**

The kinase mammalian target of rapamycin (mTOR) is a second highly conserved nutrient sensing pathway that is regulated by amino acid levels. It integrates signals from nutrients (e.g. amino acids, glucose), energy status, stress, and growth factors to mediate many processes such as cell growth, autophagy, ribosome biogenesis, and metabolism [90,91] (Figure 1.2). Rapamycin, a potent inhibitor of the mTOR pathway, is an effective immunosuppressive drug that is currently used clinically in transplantation immunosuppression therapy [92-94].
Rapamycin specifically inhibits mTOR complex 1 (mTORC1). mTORC1 is a key downstream factor in PI3K-Akt, Wnt-GSK3, and AMPK signaling pathways; all of which act primarily by impinging on and releasing the inhibitory activity of protein tuberous sclerosis complex 1 (TSC1) and TSC2 on the mTORC1 activator Rheb [90].

Figure 1.2 mTOR signaling pathway
mTOR signaling regulates translation initiation by several different inputs. Insulin, hormones, and growth factors activate the PI3K/Akt signaling cascade. Akt receives input from PDK1 and the rictor/GβL/mTOR complex (mTORC2). Energy status (AMP/ATP) modulate AMPK activity. These pathways affect TSC1/2 to regulate mTOR activity via Rheb. The raptor/GβL/mTOR complex (mTORC1) mediates the phosphorylation of 4E-BP and p70^S6K. Figure adapted from Petroulakis et al. 2006. Br J Cancer 94(2): 195-9 [95].
One of the best characterized functions of mTOR is its ability to regulate protein synthesis. Two key downstream targets of mTOR are 4E-BP1 and p70S6K, the latter of which is one of the most robust markers currently used to monitor TOR activity. Once activated by being released from TSC1 and TSC2, mTOR phosphorylates and activates the 70 kDa ribosomal protein kinase (p70S6K). Once activated, p70S6K directly phosphorylates ribosomal protein S6 (rpS6), a protein of the 40S ribosomal subunit and, by doing so, impacts cell growth by regulating the pioneer round of protein translation and the eukaryotic initiation factor 3 (eIF3) translation complex [96]. mTOR also controls translation of the 5′ capped mRNAs by directly phosphorylating the eukaryotic translation initiation factor 4E (eIF4E) binding protein (4E-BP1), resulting in dissociation from eIF4E [97]. Since mTOR phosphorylation activates p70S6K and inhibits 4E-BP1, with both events stimulating protein translation, reducing mTOR activity decreases translation. In mammals and yeast, AAD or rapamycin treatment prevents phosphorylation of mTOR and, as a result, turns off the critical component needed for protein synthesis.

Although the amino acid sensing mechanisms upstream of mTORC1 are not completely understood, TOR responds directly to intracellular microinjections of a large range of amino acids in *Xenopus laevis* oocytes; suggesting the size of the intracellular free amino acid pool, in part, regulates TOR signaling [98]. Withdrawal of amino acids from nutrient medium of CHO-IR cells results in rapid deactivation of p70S6K and dephosphorylation of 4E-BP1, which becomes unresponsive to all agonists including insulin — the addition of amino acids to basal levels in the absence of serum or insulin restores p70S6K activity and 4E-BP1 phosphorylation [99]. Moreover, the ability of amino acid withdrawal to inhibit p70S6K is only modestly decreased in TSC-null cells and is affected without alteration in Rheb-GTP charging [100,101].
Thus the pathway through which amino acids regulate mTOR is largely independent of upstream pathways (i.e. PI3K) and seems to act directly on mTORC1.

Recent studies have begun to better elucidate how amino acid levels regulate mTOR. It is proposed that amino acids signal to mTORC1 by promoting its binding to a distinct family of GTPases, the Rag GTPases, that exist in heterodimers (RagA-D) [102,103]. In the presence of amino acids, Rag GTPases promote translocation of mTORC1 to the surface of lysosomes, which also contain the mTORC1 activator Rheb [103]. Expression of a constitutively active mutant forms of Rag A or B (active GTP-bound form) in mammalian cells activate mTOR, as measured by p70S6K phosphorylation, and eliminates the requirement for amino acids in activating mTOR, whereas the dominant-negative Rag A or B (inactive GDP-bound form) inhibits mTOR in the presence of amino acids [102,103]. The Ragulator, a trimeric complex comprised of p18, p14 and MP1 proteins which functions to recruit and anchor Rag GTPases to the lysosome, interacts with Rag GTPases to form an amino acid-regulated docking site for mTORC1 on the lysosomal surface which is necessary for activation by amino acids [104]. Constitutive targeting of mTORC1 to the lysosomal surface is sufficient to render the mTORC1 pathway amino acid insensitive and independent of Rag and Ragulator, but not Rheb function [104]. Thus, to date, it is thought that the Rag-Ragulator-mediated translocation of mTORC1 to the lysosomal membrane is the key event required for amino acid signaling through mTORC1 (Figure 1.3). Another critical component in amino acid signaling to mTORC1 is the vacuolar H+-adenosine triphosphate ATPase (v-ATPase) which has been identified to function between amino acids and the nucleotide loading of Rag GTPases [105]. However, the precise mechanism by which amino acids activate Rag GTPases is still largely obscure.
Amino acid levels have been linked with mTOR activity and immune regulation in a number of studies. Reduction in amino acids levels by amino acid catabolizing enzymes (particularly IDO) expressed in dendritic cells results in a reduction in mTOR signaling, decreased T-cell proliferation, and synergistic induction of the T-regulatory (Treg)-specific transcription factor forkhead box P3 (FOXP3) with TGF–β [71]. Mimicking leucine deficiency with the competitive leucine antagonist, N-acetyl-leucine-amide (NALA) is able to mitigate T-cell proliferation and production of IL-2 and IFN-γ, and these observations coincided with a decrease in phosphorylated p70S6K levels. This suggests leucine deficiency decreases T-cell proliferation and cytokine release and does so in part through the mTOR pathway [106]. These findings highlight the central role of mTOR signaling and provide rationale for using inhibitors
of the mTOR pathway to regulate the immune system and promote tolerance in transplantation, autoimmunity, and inflammatory disease.

1.4 Targeting aminoacyl tRNA synthetases as novel immunosuppressive agents

Indeed, amino acid deprivation is a key natural mechanism by which the body regulates inflammation and immunosuppression. Strong evidence highlights the role of nutrient sensing pathways GCN2 and mTOR play in linking AAD to immune regulation. We looked to aminoacyl-tRNA synthetase inhibitors in search of candidate small molecules that can induce amino acid depleted conditions as well as activate and inhibit the GCN2 and the mTOR pathways, respectively. Aminoacyl-tRNA synthetases (aaRSs) are responsible for charging tRNA with an amino acid; this essential role in translation has earned them much attention in the past as targets for novel antimicrobial agents, however, more recent preliminary studies suggest that inhibitors of aaRSs may be immune regulating agents [107].

1.4.1 Aminoacyl tRNA synthetase inhibitors

Amino acids required for translation of mRNA are delivered to the ribosome esterified to the 3’ ends of tRNA. The aminoacylation of tRNAs is catalyzed by aaRSs, which have the ability to identify and discriminate their unique cognate pair of amino acids and tRNAs from the many other similar molecules that exist within the cell [108]. This reaction, called aminoacylation or “charging” of tRNAs, is carried out in a highly specific two-step reaction (Figure 1.4). In the first step, the amino acid is activated by attacking a molecule of ATP at the α-phosphate, leading to the formation of an enzyme bound mixed anhydride intermediate, aminoacyl-adenylate (aa-AMP), and an inorganic pyrophosphate. In the second step, the amino
acid moiety is transferred to the 2’ or 3’ hydroxyl of the terminal ribose of a cognate tRNA yielding an aminoacyl tRNA and AMP [108]. In order to get incorporated into a growing peptide chain, amino acids are required to undergo this reaction to create an aminoacyl-tRNA.

1) \[\text{aaRS} + \text{aa} + \text{ATP} \rightarrow \text{aaRS} \cdot (\text{aa-AMP}) + \text{PP}_i\]

2) \[\text{aaRS} \cdot (\text{aa-AMP}) + \text{tRNA} \rightarrow \text{aaRS} + \text{aa-tRNA} + \text{AMP}\]

**Figure 1.4 Enzymatic mechanism of aaRS**
Aminoacylation is a two-step reaction. In the first step, the amino acid is activated by attacking a molecule of ATP at the α-phosphate, leading to the formation of aminoacyl-adenylate (aa-AMP), and an inorganic pyrophosphate (PP$_i$). In the second step, the amino acid moiety is transferred to the 2’ or 3’ hydroxyl of the terminal ribose of a cognate tRNA yielding an aminoacyl tRNA and AMP.

Because of the essential role aaRSs have within the cell, inhibitors of these enzymes have been actively studied as potential antibiotics and anti-viral agents [109-111]. In all eukaryotes from yeast to mammals, inhibiting aaRSs increases the levels of uncharged tRNA within a cell which directly activates the GCN2 pathway. Because inhibiting aaRSs prevents specific amino acids from adhering to their tRNA, these free amino acids within the cell get rapidly catabolised and utilized in other cellular processes [112]. In more recent years, studies have begun to elucidate the effects of aaRS inhibitors on human cells. aaRS inhibitors have been shown to decrease the proliferation of immune cells in a mixed lymphocyte reaction [107] and suppress p70$^{	ext{S6K}}$ activity [113]. Moreover, aaRS inhibitors may be utilizing the GCN2 pathway much like natural amino acid catabolizing enzymes, as tryptophanol, a competitive inhibitor of TrpRS, has been shown to activate GCN2 in T-cells [78]. This activation of GCN2 is most likely due to the increase in tRNAs that arises when an aaRS is blocked; tRNAs are unable to couple with their
cognate amino acid. These findings suggest aaRS inhibitors may hold promise as novel immunosuppressive agents and require further investigation.

1.4.2 Borrelidin, a threonyl-tRNA synthetase inhibitor

Borrelidin is an 18-membered macrolide from the *Streptomyces* species with antimicrobial and antiangiogenic properties and is a known inhibitor of bacterial and eukaryotic threonyl-tRNA synthetase (ThrRS). Borrelidin is a non-competitive inhibitor of threonine that binds to a hydrophobic region proximal to the ThrRS active site, and when bound it induces a conformation change that prevents threonine activation [114]. Borrelidin was first discovered in the 1950’s as an anti-microbial agent, but further clinical development of borrelidin was not pursued due to the fact it potently inhibits mammalian threonyl-tRNA synthetase. In more recent years, borrelidin has come to the forefront as an antiangiogenic agent with activity at nanomolar concentrations [115-117]. This makes borrelidin highly attractive because drug candidates with low nanomolar activity normally have a higher degree of selectivity and affinity for their target and are less likely to have off-target effects [118].

1.5 Hypothesis, objectives, and specific aims

1.5.1 Objectives and hypothesis

The overall aim of this thesis project was to determine if borrelidin, a ThrRS inhibitor holds potential as an immunosuppressive and/or anti-inflammatory agent. Aberrant immune responses require clinical intervention to prevent tissue damage and organ dysfunction; unfortunately, many of these clinical interventions have undesired side effects. Therefore,
development of novel therapeutic agents with different mechanisms of action would be immensely beneficial. Mounting evidence in *in vitro* and *in vivo* studies implicate amino acid depletion as a natural mechanism which the body uses to elicit localized immunosuppression. However, the role of aminoacyl-tRNA synthetase inhibitors in regulating immune responses is largely unknown. Since inhibitors of aaRSs limit the cell’s access to specific amino acids and thereby create an amino acid limiting environment, a deeper investigation of aaRS inhibitor-induced amino acid deprivation and its ability to regulate immune cell function is needed. Thus, we hypothesized that **borrelidin, a ThrRS inhibitor, is a potent immunosuppressive and anti-inflammatory agent.** This hypothesis was investigated through the following specific aims:

**Specific Aim 1:** To determine if borrelidin can suppress primary T-cell activation and function *in vitro*

**Specific Aim 2:** To study the effect of borrelidin on inhibiting cytokine release by macrophages

1.5.2 Experimental plan

A detailed description of the animals, materials, experimental methods and techniques used in this study are provided in the material and methods sections of the following chapters (Chapters 2.2 and 3.2). Here is a brief overview of the research plan:

**Specific Aim 1:** To determine if borrelidin can suppress primary T-cell activation and function *in vitro.* To address Specific Aim 1, primary mouse leukocytes were isolated from the spleen of mice and stimulated in one of three ways: mixed leukocyte reaction, anti-CD3/anti-CD28 crosslinking, or phorbol 12-myristate 13-acetate (PMA) + ionomycin. The ability of T-cells to
proliferate in the presence of borrelidin was monitored using [3H]-thymidine as a marker of DNA synthesis. T-cell cytokines IL-2, IL-4 and IFN-γ were monitored using ELISA and T-cell cell surface activation markers, CD25 and CD69, were examined with flow cytometry. In parallel, intracellular staining and flow cytometry was performed to monitor activity of the GCN2 and mTOR pathways.

**Specific Aim 2**: To study the effect of borrelidin on inhibiting cytokine release by macrophages. To address Specific Aim 2, lipopolysaccharide (LPS) induced cytokine production was monitored in macrophages in the presence or absence of borrelidin using ELISA and a multiplex immunoassay approach. The ability of borrelidin to suppress cytokine production was further examined by quantitating mRNA transcript levels using Q-PCR and intracellular cytokine protein levels by blocking the golgi apparatus with brefeldin A, followed by ELISA analysis. In parallel, intracellular staining and flow cytometry was performed to monitor activity of the GCN2 and mTOR pathways.
CHAPTER 2. Borrelidin: A novel potent immunosuppressive agent

2.1 Introduction

Despite excellent short-term graft and patient survival rates, organ transplantation continues to face challenges in poor long-term graft survival in part from the major side effects associated with long-term immunosuppressive therapy such as nephrotoxicities caused by calcineurin inhibitors. Thus, there is currently a large unmet medical need for development of new and more potent immunosuppressive agents with unique or novel mechanisms of action that show high synergistic efficiency with current agents in order to minimize any associated toxicities. Development of new agents may lead to a higher rate of graft tolerability with fewer side effects, facilitate long-term graft survival, and improve patient quality of life and survival.

A natural mechanism the body uses to mediate immunosuppression is through the expression of immunoregulatory enzymes, such as arginase and indoleamine 2,3-dioxygenase (IDO) [31]. Arginase and IDO exert localized immunomodulatory effects on T-cells through catabolizing the amino acids arginine and tryptophan, respectively, to create a low amino acid environment. Immune cells are exquisitely sensitive to amino acid deprivation compared to other cells in the body. Indeed, IDO is important in the regulation of many different types of immune responses, such as preventing semi-allogeneic fetus rejection [51] and in the pathological conditions including neoplasia [60,61], chronic infection [31], asthma [58] and autoimmune diseases [53].

Recent studies suggest that the stress-responsive kinase, general control non-depressible 2 (GCN2) signaling pathway enables T-cells to sense and respond to low amino acid conditions generated by IDO [78]. Mechanistically, amino acid depletion (AAD) can cause
a rise in the levels of uncharged transfer RNAs (tRNAs) in cells which activate the regulatory
domain of GCN2, triggers its kinase activity, and initiates the downstream signaling. Activation
of GCN2 results in phosphorylation of eukaryotic initiation factor 2α (eIF2α) [119] leading to
inhibition of general protein synthesis while simultaneously stimulating synthesis of specific
stress response proteins of the integrated stress response (ISR) [84,120]. AAD also antagonizes
a second well characterized highly conserved nutrient sensing pathway, mammalian target of
rapamycin (mTOR) which is currently being used as a clinical target for transplant
immunosuppression therapy [92-94].

In search of candidate small molecules that can induce the low amino acid
microenvironment we see regulating natural immunosuppression, we looked to aminoacyl-tRNA
synthetase inhibitors. Aminoacyl-tRNA synthetases (aaRSs) are key enzymes that catalyze the
aminoacylation of specific amino acids onto their cognate tRNAs and thus play a pivotal role in
protein biosynthesis [121]. Consequently, inhibition of these enzymes leads to an accumulation
of uncharged tRNAs and inhibition of cell proliferation. The development of this class of agents
has historically been actively pursued as potential antibiotics and anti-viral agents [109,110,122].
However, in more recent years, aaRS inhibitors have been shown to decrease the proliferation of
immune cells in a mixed lymphocyte reaction [107] and suppress p70^{S6K} activity, a well-known
downstream marker for mTOR activity [113] suggesting aaRS inhibitors may hold promise as
novel immunosuppressive agents.

One well characterized natural occurring ThrRS inhibitor is the small molecule nitrile-
containing macrolide borrelidin. Borrelidin was first discovered in the 1950’s as an anti-
microbial and anti-viral agent; however, further clinical development of borrelidin as an
antibiotic was discontinued due to the fact that it also potently inhibited mammalian ThrRS
[115]. In more recent years, borrelidin has come to the forefront as an antiangiogenic agent with
activity at nanomolar concentrations [116,117]. Due to the increasing evidence that aaRS inhibitors are immunosuppressive and that borrelidin is able to inhibit mammalian ThrRS, we hypothesize that borrelidin holds utility as a novel immunosuppressive agent. Therefore, the aim of the present study is to investigate whether borrelidin has the potential to suppress T-cell activation and function. Our results indicate that borrelidin potently and more effectively inhibits primary T-cell activation and proliferation. These findings support the notion that the ThrRS inhibitor, borrelidin, can be utilized as an immunosuppressive agent through harnessing a naturally occurring immunosuppression mechanism used to regulate immune cell function.

2.2 Materials and methods

2.2.1 Ethical statement

Following written informed consent, primary human skin fibroblasts were established from neonatal foreskin with the approval of the Institutional Ethics Committee of the University of British Columbia (protocol number: H05_70537). All animal experimental procedures were carried out in compliance with the guidelines of the Canadian Council for Animal Care and were approved by the institutional Animal Care Committee of the University of British Columbia (protocol number: A09_0397). Spleens were collected from surplus mice from various protocols at the Jack Bell Research Centre; these animals were not treated in any way and since they were surplus mice they would otherwise have been euthanized.
2.2.2 Cell culture

Human fibroblasts were isolated from human foreskin as previously described [123] and maintained in Dulbecco’s Modified Eagle medium (DMEM) (Hyclone, Ottawa, ON) containing 10% fetal bovine serum (FBS) (Hyclone, Ottawa, ON). The human keratinocyte cell line, HACAT, were cultured in DMEM containing 10% FBS. For both fibroblasts and HACAT cells, at 70% confluency, cells were detached with 0.25% trypsin/EDTA (Gibco, Carlsbad, CA), and split at a 1:10 ratio. Primary splenocytes were harvested from either BALB/c, CD1, or C57BL/6 mice spleens. Briefly, spleens were collected and a single cell suspension was prepared by gentle mechanical disruption of the spleens through a 40 µM cell strainer (BD, Biosciences Mississauga, ON) followed by Histopaque-1077 (Sigma Aldrich Inc., St. Louis, MO) density gradient centrifugation. Splenocytes at the interface were collected and maintained in complete growth media (RPMI 1640 (Hyclone, Ottawa, ON), containing 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco, Carlsbad, CA), 50 µM beta-mercaptoethanol (Sigma-Aldrich, Inc., Saint Louis, MO), 1 mM sodium pyruvate (Gibco, Carlsbad, CA), and 2 mM L-glutamine (Gibco, Carlsbad, CA)) supplemented with 10% FBS. In experiments that required the absence of tryptophan, cells were washed twice with phosphate buffered saline (PBS) then resuspended in custom made tryptophan deficient RPMI 1640 media containing 10% dialyzed serum (Hyclone, Ottawa, ON). Cells were cultured at 37° C in an atmosphere of 5% CO₂ and 95% air.

2.2.3 Preparation of borrelidin

Borrelidin was purchased from Axxora Life Sciences Inc. (San Diego, CA) and reconstituted in dimethyl sulfoxide (DMSO) (Sigma Aldrich Inc., St. Louis, MO) at 1mg/ml and
stored at -20°C until use. Borrelidin was diluted in culture medium prior to use. DMSO without borrelidin was used as a control.

### 2.2.4 Two-way allogeneic mixed leukocyte reaction

Mixed leukocyte reactions were used to monitor the activation of unprimed T lymphocytes in response to stimulation by cell associated allogeneic MHC antigens. T-cell proliferation was monitored by [\(^3\)H] -thymidine incorporation into the DNA of growing cells as a measure of DNA synthesis. Splenocytes from C57BL/6 (responder) and either CD1 or BALB/c (stimulator) mice were each seeded at 2x10^5 cells/well in a 96 well plate in RPMI complete growth media in the presence of borrelidin for 48 hours. In some cases, 1 mM L-threonine (Sigma Aldrich Inc., St. Louis, MO) was added to the RPMI complete media prior to addition of stimulator and responder cells. Alternatively, CD4^+ and CD8^+ T-cells were purified using STEMCELL Technologies’ CD4^+ and CD8^+ EasySep Cell Separation T-cell Enrichment Kit (STEMCELL Technologies, Vancouver, BC) and seeded at 4x10^4 cells/well with 5x10^5 cells/well mitomycin C treated BALB/c splenocytes in RPMI complete growth media in the presence of borrelidin. After 48 hours 1 µCi/well of [\(^3\)H]-thymidine (Perkin Elmer, Waltham, MA) in 25 µl of fresh media was spiked into each well for an additional 18 hours. Incorporation of [\(^3\)H]-thymidine was measured in the harvested cells using MicroBeta Liquid Scintillation Counter (Wallac).
2.2.5[^3]H Thymidine incorporation assay

Proliferation of cells was analysed by[^3]H thymidine incorporation as a measure of DNA synthesis. Fibroblast or HACAT cells were seeded at 3,000 cells/well and 5,000 cells/well, respectively, in a flat bottom 96-well plate and stimulated to proliferate with growth factors in the FBS in the presence (0 - 4 μM) or absence of borrelidin for 48 hours. Alternatively, B-cells in a primary mixed splenocytes population were stimulated to proliferate with 10 μg/ml anti-IgM (Jackson ImmunoResearch, West Grove, PA) for 48 hours. 1 μCi/well of[^3]H-thymidine in 25 μl of fresh media was spiked into each well for an additional 18 hours. Incorporation of[^3]H-thymidine was measured in the harvested cells using MicroBeta Liquid Scintillation Counter.

2.2.6 Induction and measurement of T-cell responses

Splenocytes were preincubated at 37°C with borrelidin (0, 4.1, 10.2 or 20.4 nM) or tryptophan-deficient media for 30 min prior to stimulation by plate bound anti-CD3 (1 μg/ml) (clone: 2C11 BD, Biosciences Mississauga, ON) + anti-CD28 (2 μg/ml) (BD, Biosciences Mississauga, ON) crosslinking in a 24-well plate, at a cell density of 1x10^6 cells/well. 24 hours later, the levels of IL-2, IL-4, and IFN-γ produced by activated T-cells were measured in the conditioned media using the mouse enzyme-linked immunosorbent assay (ELISA) system. Activated cells were collected and washed 2 times in PBS + 2% FBS and then stained using a combination of anti-CD4, anti-CD25, and anti-CD69 antibodies for 45 min on ice. Cells were then washed an additional 2 times with PBS + 2% FBS and then analyzed using a Becton Dickinson FACS Canto II flow cytometer. For western blot experiments, nylon wool (Wako Chemicals USA Inc., Richmond, VA) enriched primary T-cells were stimulated for 24 hours with 50 ng/ml PMA (Sigma-Aldrich, Inc. Saint Louis, MO), and 500 ng/ml ionomycin (Sigma-
Aldrich, Inc. Saint Louis, MO), at 8x10^6 cells per 10 cm² plate in a total of 4ml of RPMI complete growth media. Borrelidin (0, 20.4, 40.8 nM) was added to the stimulated cells for 3 hours. For intracellular staining a single cell suspension of splenocytes were activated by 50 ng/ml PMA and 500 ng/ml ionomycin in the presence of borrelidin (0, 10.2, 20.4, 40.8 nM), 20 nM rapamycin or absence of tryptophan for 3 hours.

2.2.7 Enzyme-linked immunosorbent assay

The levels of IL-2, IL-4, and IFN-γ produced by activated mouse T-cells were measured using their respective mouse BD OptEIA Set ELISA system (BD, Mississauga, ON) according to manufacturer’s protocols.

2.2.8 Analysis of CD25 and CD69 cell surface expression

The expression of cell surface markers was evaluated by flow cytometry. After 24 hours of stimulation, splenocytes were harvested and washed twice with PBS. Cells were stained for 45 minutes on ice in the dark with phycoerythrin (PE)-conjugated anti-mouse CD4 (BD, Mississauga, ON) and either allophycocyanin (APC) conjugated anti-mouse CD25 (eBioscience, San Diego, CA) or fluorescein isothiocyanate (FITC) conjugated anti-mouse CD69 (BD, Mississauga, ON). Cells were then washed with FACS buffer (2% FBS in PBS) to remove excess stains. Samples were then analyzed by a FACSCanto II flow cytometer (Becton Dickinson, San Jose, CA) using FlowJo software (FlowJo Ashland, OR). The analysis of the CD25 and CD69 populations was based on CD4-positive gated populations. 20,000 events were collected for each sample based on the CD4-positive gated population with each event
representing a single cell or particle. Gates for CD4, CD25 and CD69 positive cells were set based the unstimulated single stained controls.

2.2.9 Western blot

Cells were lysed in NP40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 2 mM EDTA, 1% TritonX-100) (Fischer Scientific Ottawa, ON) and 1x Protease Inhibitor Cocktail (Roche, Laval, QC), for 30 min on ice. Cell lysates were centrifuged for 20 min at 4°C at 13,000 RPM to remove insoluble cellular debris. Total protein quantification was performed on the supernatant using a BCA Protein Assay (Thermo Fisher). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins according to size. The SDS-PAGE was transferred to a nitrocellulose membrane and blocked for 1 hour in 5% bovine serum albumin (BSA) (Roche, Laval, QC) TBST at room temperature with gentle agitation. Primary antibodies were incubated shaking over night at 4°C in 1% BSA TBST at 1:1000 phospho-eIF2α (Ser 51) (Cell Signaling Inc., Danvers, MA, USA) and eIF2α (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), 1:500 C/EBP homologous protein (CHOP/GADD153) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Membranes were washed 3 times in TBST and incubated with an anti-rabbit secondary antibody (Dako Diagnostics Canada Mississauga, ON) conjugated to horseradish peroxidase (HRP). Chemiluminescence was performed with Amersham’s ECL kit.
2.2.10 Analysis of intracellular phosphorylated eIF2α

Cells were first stained on ice for 1 hour with a FITC-conjugated anti-CD3 antibody (BD, Mississauga, ON). Cells were then fixed using BD Cytofix, following the manufacturer’s instructions, and permeabilized in 0.2% triton-X in PBS for 20 minutes at room temperature. After permeabilization, cells were washed once in TPBS (PBS + 0.05% Tween 20) and blocked with 5% normal goat serum (NGS) in TPBS for 20 minutes at room temperature. Cells were then incubated at 37°C for 1 hour with a 1:100 final dilution of anti phospho-eIF2α (Ser 51) antibody (Cell Signaling Technology, Danvers, MA) in 1% BSA/1%NGS in TPBS. The secondary antibody was an APC-conjugated anti-rabbit IgG (H+L) (Jackson ImmunoResearch West Grove, PA). Cells were analyzed using Becton Dickinson FACS Canto II flow cytometer and FlowJo software. The analysis of the phospho-eIF2α population was based on CD3-positive gated population. 10,000 events were collected for each sample based on the CD3-positive gated population. Gates for CD3 and phospho-eIF2α were set based on the unstimulated single stained controls.

2.2.11 Analysis of intracellular phosphorylated ribosomal protein S6 (rpS6)

Cells were first stained on ice for 1 hour with an anti-CD3 antibody (BD, Mississauga, ON) followed by 1 hour on ice with an Alexa 680-conjugated anti-rabbit secondary antibody (Dako Diagnostics Canada Mississauga, ON). Cells were then fixed and permeabilized by BD Cytofix/Cytoperm (BD, Mississauga, ON) for 20 min on ice. The alexa488-conjugated anti-phospho rpS6 (Ser235/236) (Cell Signaling Inc., Danvers, MA, USA) was diluted 1:10 in BD Perm/Wash (BD, Mississauga, ON) and incubated overnight at 4°C. Cells were analyzed using Becton Dickinson FACS Canto II flow cytometer and FlowJo software. The analysis of the
phospho rpS6 expression was based on CD3-positive gated population. 10,000 events were collected for each sample based on the CD3-positive gated population. Gates for CD3 and phospho-rpS6 were set based on the unstimulated single stained controls.

2.2.12 Statistical analysis

Statistical analyses were performed using Student’s $t$ test. Significance was assessed at the 95% confidence level.
2.3 Results

2.3.1 Borrelidin inhibits T-cell proliferation

First we tested borrelidin on primary T-cell proliferation. To monitor T-cell growth we performed an allogeneic mixed leukocyte reaction (MLR), which is a robust in vitro method for assaying T-cell proliferation [124]. T-cell proliferation was measured by $[^3H]$ - thymidine incorporation as a measure of DNA synthesis. In Figures 2.1A and 2.1B, CD4$^+$ T-cells and CD8$^+$ T-cells, respectively, were purified from C57BL/6 splenocytes and challenged with mitomycin C treated BALB/c splenocytes. Results showed borrelidin was able to potently and dose dependently inhibit proliferation of purified T-cells (Figure 2.1A-B) and T-cells in a two-way MLR (black line, Figure 2.1C-E) with a half maximal inhibitory concentration (IC$_{50}$) of 3.3 nM. The ability of borrelidin to suppress the proliferation and function other cell types: primary human fibroblasts, mouse B-cells, HACAT cells, primary mouse macrophages, and mouse bone-marrow derived mast cells (BMMC) was also examined (Table 2.1). Borrelidin effectively suppressed proliferation of anti-IgM stimulated B-cells, with a 9-fold higher tolerance (IC$_{50}$ of 29 nM) toward the anti-proliferative activity of borrelidin (Figure 2.1C). Similarly, primary fibroblasts from foreskin and HACAT cells showed an approximate 300-fold higher tolerance (IC$_{50}$ of 1000 nM and 850 nM, respectively) toward the anti-proliferative activity of borrelidin (Figure 2.1D and Figure 2.1E). Borrelidin also suppressed the release of TNF$\alpha$ from anti-mouse IgE stimulated bone marrow derived mast cells (BMMCs) with an IC$_{50}$ of 960 nM and TNF$\alpha$ from LPS stimulated primary splenocytes with an IC$_{50}$ of 22 nM (see Chapter 3). Cumulatively, these data suggest borrelidin is a potent suppressor of T-cell growth and more effectively inhibits T-cells as compared with other cell types.
Figure 2.1 Borrelidin inhibits T-cell proliferation
A one-way mixed leukocyte reaction (MLR) was performed between CD4\(^+\) (A) and CD8\(^+\) (B) purified T-cells and mitomycin C treated BALB/c splenocytes in increasing concentrations of borrelidin as indicated. A two-way MLR was performed between BALB/c and CD1 mice (black line in both panel C-E) or (C) 10 µg/ml anti-IgM activated splenocytes (D) primary fibroblasts, or, (E) HACAT cell line were cultured (grey line) in increasing concentrations of borrelidin as indicated. After 48 hours 1 µCi/well of \[^3\]H\]-thymidine was spiked into each well for 18 hours. Incorporation of \[^3\]H\]-thymidine into DNA as an index of proliferation was measured in the harvested cells using a MicroBeta Liquid Scintillation Counter (Wallac). Statistical significance (p < 0.05*) was determined by comparing the condition with the untreated stimulated condition (0 nM borrelidin, MLR stimulated).
Table 2.1 Immunosuppressive activity of borrelidin on different cell types

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>IC\textsubscript{50} (nM)</th>
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<tr>
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<td>Macrophages</td>
<td>22</td>
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<tr>
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<td>Mast cells</td>
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<tr>
<td>Fibroblasts</td>
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2.3.2 Borrelidin inhibits production of cytokines by activated primary T-cells

To assess the effects of borrelidin on T-cell stimulated cytokine production, splenocytes were stimulated with anti-CD3/anti-CD28 — antibodies that mediate T-cell specific activation. ELISA was performed to measure the level of cytokines IL-2, IL-4, and IFN-\(\gamma\), released in the culture supernatant of borrelidin treated activated T-cells. Our results showed that the stimulation of mouse splenocytes with anti-CD3 and anti-CD28 resulted in production and secretion of IL-2, IL-4, and IFN-\(\gamma\) into the culture medium (Figure 2.2). A significant reduction in the levels of IL-2, IL-4, and IFN-\(\gamma\) was observed in activated splenocytes treated with 20.4nM borrelidin. Additionally, IL-2 and IFN-\(\gamma\) continued to show a significant reduction in extracellular levels at 10.2 nM borrelidin. No significant difference was detected between the decrease in cytokines due to borrelidin treatment (20.4 nM) and the decrease in cytokines due to cell stimulation in the absence of tryptophan (control).
Figure 2.2 Borrelidin inhibits cytokine release upon stimulation.
Primary splenocytes were stimulated with anti-CD3 (1 µg/ml) and anti-CD28 (2 µg/ml) in the presence of the indicated concentration of borrelidin. After 24 hours, IL-2, IL-4 and IFN-γ levels in the cell culture supernatant were determined by ELISA. Statistical significance (p < 0.05*) was determined by comparing the condition with the untreated stimulated condition (0 nM borrelidin, anti-CD3/anti-CD28 stimulated).

2.3.3 Borrelidin inhibits upregulation of T-cell activation markers

CD25, the alpha chain of the IL-2 receptor, and CD69, an early activation antigen, are cell surface markers that are upregulated on T-cells upon T-cell activation. Flow cytometric analyses showed that cell surface expression of CD25 and CD69 was upregulated in mouse CD4-positive T-cells after 24 hour stimulation with anti-CD3 (1 µg/ml) and anti-CD28 (2 µg/ml) (Figure 2.3). Significant reduction of these cell surface proteins was found in activated splenocytes treated with borrelidin (20.4 nM). No significant difference was detected between the decrease in cell surface expression of CD25 and CD69 due to borrelidin treatment and the decrease in cell surface expression of CD25 and CD69 due to stimulation in the absence of tryptophan. Representative histograms at each concentration of borrelidin are shown in Figure 2.3B.
Figure 2.3 Effects of borrelidin on CD69 and CD25 cell surface expression.
Primary splenocytes were stimulated with anti-CD3 (1 µg/ml) and anti-CD28 (2 µg/ml) in the presence of the indicated concentration of borrelidin. After 24 hours, the levels of cell surface activation markers CD25 or CD69 in a CD4-positive gated population were independently monitored by flow cytometry. (A) Average mean fluorescence intensity was determined from CD4-positive gated populations from three independent experiments. Representative histograms are shown in panel B with CD25 (top) and CD69 (bottom) positive staining shown in blue and the unstimulated control shown in red. Statistical significance (p < 0.05*) was determined by comparing the condition with the untreated stimulated condition (0 nM borrelidin, anti-CD3/anti-CD28 stimulated)
2.3.4 Borrelidin stimulates phosphorylation of eIF2α in T-cells and inhibits phosphorylation of ribosomal protein S6

Because IDO expression is known to activate GCN2 and induce expression of C/EBP homologous protein (CHOP) [78], we wondered if borrelidin could also signal through the GCN2 and mTOR pathways and induce expression of CHOP. We stimulated primary splenocytes with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) and ionomycin (500 ng/ml) in the presence or absence of borrelidin, and monitored phosphorylation levels of eIF2α and protein levels of CHOP. We also monitored phosphorylation levels of ribosomal protein S6 (rpS6), a robust downstream target of mTOR and is widely used to monitor the activation state of mTOR [125]. Treatment of activated T-cells with 20.4 nM and 40.8 nM borrelidin induces an increase in eIF2α phosphorylation and CHOP expression as compared to the stimulated T-cells treated in the absence of borrelidin (Figure 2.4A and Figure 2.4B). In both cases the total levels of eIF2α did not change upon treatment. Intracellular staining for phosphorylated eIF2α confirms borrelidin is indeed significantly inducing eIF2α phosphorylation (Figure 2.4C). The phosphorylated level of rpS6 was significantly decreased by 40.8 nM borrelidin as compared with the untreated stimulated T-cells (Figure 2.4D). These data suggests borrelidin treatment results in activation of the GCN2 pathway and inhibition of the mTOR pathway in T-cells.
Figure 2.4 Borrelidin induces eIF2α phosphorylation and inhibits phosphorylation of ribosomal protein S6

Nylon wool enriched primary T-cells from purified splenocytes were stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin. After 24 hours, borrelidin (0-40.8 nM) was added to the cultures. Cell lysates were made in NP40 lysis buffer at 3 hours after borrelidin addition. Both eIF2α (A) and CHOP (B), a direct downstream maker of GCN2 activation were upregulated in the presence of borrelidin. Representative immunoblots are shown. (C,D) Primary splenocytes were stimulated with 50ng/ml PMA and 500ng/ml ionomycin in the presence of the indicated concentration of borrelidin. After 3 hours, the levels of intracellular phosphorylated eIF2α (C) and phosphorylated rpS6 (D) were monitored by flow cytometry and the average mean fluorescence intensity was collected from CD3-positive gated populations. Ratio of the mean fluorescence intensity (MFI) for each treatment was compared against the 0nM borrelidin, PMA/Ionomycin stimulated to determine the fold increase or decrease in phosphorylation states. Statistical significance (p < 0.05*) was determined by comparing the condition with the untreated stimulated condition (0 nM borrelidin, 50 ng/ml PMA + 500 ng/ml ionomycin stimulated)
2.3.5 L-threonine can rescue cells from borrelidin immunosuppressive activity

Since borrelidin has been shown to be a non-competitive inhibitor of ThrRS in respect to ATP and threonine [114,126], we asked whether the potent suppressive effects of borrelidin on T-cell proliferation could be reversed by the addition of L-threonine in a MLR. 1 mM L-threonine was added into the culture medium during a MLR and the capacity of T-cells to undergo proliferation was measured by $[^3]$H thymidine incorporation as a measure of DNA synthesis. Our results show that L-threonine was able to significantly rescue borrelidin’s potent suppression of T-cell proliferation as noted by a significant increase in $[^3]$H thymidine incorporation at borrelidin concentrations over 5.1 nM (Figure 2.5).

![Figure 2.5 L-Threonine can rescue cells from borrelidin-induced inhibition of T-cell proliferation](image)

**Figure 2.5 L-Threonine can rescue cells from borrelidin-induced inhibition of T-cell proliferation**

A two-way mixed leukocyte reaction (MLR) was performed between splenocytes derived from BALB/c and C57BL/6 mice in the presence (2.6-40.8 nM) or absence (0 nM) borrelidin (black) and with the addition of 1 mM L-threonine (grey). After 48 hours, 1 μCi/well of $[^3]$H-thymidine was added into each well for 18 hours. Incorporation of $[^3]$H-thymidine into DNA as an index of proliferation was measured in the harvested cells using MicroBeta Liquid Scintillation Counter (Wallac). Statistical significance (p < 0.05*) was determined by comparing the condition containing 1mM L-threonine to the condition without 1mM L-threonine.
2.4 Discussion

T lymphocytes play a pivotal role in the pathogenesis of cell-mediated autoimmune diseases and immune-mediated rejection of transplanted allogeneic organs and tissues. Development of new immunosuppressive agents may lead to higher rate of graft tolerability, fewer side effects and improved patient quality of life. Previous studies have suggested aminoacyl tRNA synthetase inhibitors exhibit immunosuppressive activity [107]; however, the effect of borrelidin, a ThrRS inhibitor, on T lymphocytes is still largely unknown. Therefore, in the present study we aimed to investigate whether borrelidin, has the potential to suppress T-cell activation and function as a novel immunosuppressive agent.

Previously, Van de Vijver et al. showed that a panel of chemical mimics of aminoacyl tRNA synthetase reaction-intermediates exhibited immunosuppressive activity in mixed lymphocyte reactions exhibiting IC$_{50}$ values ranging from 0.1-10 µM with the most potent at 80 nM [107]. Because of these early findings, our first step was to examine the effect of borrelidin on allogeneic MLR induced T-cell proliferation in vitro. As a result, borrelidin inhibited T-cell proliferation dose-dependently with a half maximal inhibitory concentration (IC$_{50}$) of 3.3 nM with nearly complete inhibition at 20.2 nM. The discrepancy in the potency of different ThrRS inhibitors is likely due to differences in the binding affinity and interaction site of borrelidin as compared to reaction-intermediate mimics. Borrelidin binds a hydrophobic noncatalytic domain near the active site of ThrRS which impairs catalytic conformational changes resulting in reduced binding of ATP and Thr [114], while the reaction-intermediate mimics tested by Van de Vijver et al. directly bind and inhibit the active site of their respective aaRSs. Moreover, as to T-cell activation, borrelidin also remarkably inhibited the anti-CD3/anti-CD28 stimulated upregulation of CD25 and CD69 in CD4$^+$ primary T-cells, which are T-cell activation markers. Consistent with the inhibitory effect of borrelidin on T-cell proliferation and upregulation of cell
surface activation markers, borrelidin dose-dependently inhibited anti-CD3/anti-CD28 stimulated production of IL-2, IL-4, and IFN-γ in activated T-cells. Much lower levels of IL-4 were observed as compared to IL-2 and IFN-γ, which was anticipated as anti-CD3/anti-CD28 activation of unprimed T-cells has been shown to produce only moderate levels of IL-4 at 24 hours of stimulation [127]. Since the production of IL-2 is an essential component in the activation and maintenance of T-cell proliferation, the decrease in IL-2 production may contribute to the borrelidin-induced suppression of MLR stimulated T-cell proliferation. Cumulatively these findings indicate that borrelidin can exert an immunosuppressive effect through its inhibition of T cell proliferation, activation and cytokine production.

It has recently been suggested that in murine T-cells, the GCN2 kinase pathway detects and responds to the low amino acid conditions generated by IDO [78]. In this study, by detecting the phosphorylated levels of eIF2α, the direct downstream substrate of GCN2, we have shown that there is a positive correlation between increasing dose of borrelidin and eIF2α phosphorylation. We have also shown under the same experimental conditions that the levels of CHOP expression are higher in borrelidin treated cells compared to the untreated cells and that increasing borrelidin concentrations inhibits phosphorylation of rpS6. Our results suggest borrelidin is activating the GCN2 pathway and inhibiting the mTOR pathway in T-cells and this may be one of the underlying mechanisms by which borrelidin is eliciting its immunosuppressive activity. Consistent with our findings, activation of the amino acid starvation response has been shown to inhibit Th17 differentiation and protect mice against Th17-associated experimental encephalomyelitis [87]. Furthermore, it has been speculated that up-regulation of CHOP induced by eIF2α phosphorylation as a result of endoplasmic reticulum stress may in part be responsible for impaired lymphoproliferative responses after measles virus infection [128]. Our findings that the GCN2 and mTOR pathways are involved in sensing the
conditions created by borrelidin support the overall understanding how the cell responds to amino acid deprivation to regulate immune function.

In contrast to T-cells, we found primary fibroblasts, HACAT cells, and B-cells exhibited a significantly higher proliferative IC$_{50}$ values suggesting borrelidin is a more effective inhibitor of T-cells. This more selective sensitivity was expected as T-cells are exquisitely sensitive to amino acid deprivation [129,130]. The observed resistance to borrelidin-induced inhibition of proliferation in fibroblasts and HACAT cells could be accounted for by IMPACT, a known inhibitor of GCN2, which is highly and constitutively expressed in skin cells, while absent in immune cells [131,132]. Recently it was suggested that high expression of protein IMPACT homolog in non-immune cells such as skin cells, acts as a protective mechanism against IDO-induced GCN2 activation, therefore, making them more resistant to the amino acid-deprived environment caused by IDO [131]. As such, we speculate that IMPACT in fibroblasts and HACAT may be playing a role in protecting these cells from borrelidin-induced GCN2 activation. However, B-cells exhibited a higher proliferative IC$_{50}$ as well, which might reflect utilization of different pathways of cell activation, and as mentioned above, the decrease in IL-2 production may contribute to the borrelidin-induced suppression of T-cell proliferation. It follows that localized administration of borrelidin, such as in organ transplantation, may utilize the selective inhibition of T-cells by borrelidin.

The macrolide antibiotic borrelidin acts as non-competitive inhibitor of the threonyl tRNA synthetase with respect to threonine and inhibits aminoacylation of threonine tRNAs [114,126]. Since IDO-mediated immunosuppression can be reversed by addition of excess tryptophan [78], we asked whether the potent suppressive effects of borrelidin on T-cell proliferation could be reversed by the addition of L-threonine in a MLR. Our results show that borrelidin mediated immunosuppression can be reversed by addition of excess threonine.
Therefore, the ability to reverse the immunosuppressive actions of borrelidin at will, using a relatively innocuous agent such as threonine, makes borrelidin highly appealing as a potential therapeutic agent.

In summary, we have demonstrated that borrelidin inhibits primary T-cell activation and proliferation. These findings extend our understanding of the immunosuppressive effect of borrelidin; they also support the potential of aminoacyl tRNA synthetase inhibitors as immunosuppressive agents used to treat autoimmune diseases and prevent graft rejection.

3.1 Introduction

Inflammation is the body’s normal physiological response to tissue injury, infection or foreign substances. Excessive or dysregulated inflammation results in chronically elevated levels of inflammatory mediators which are considered to contribute to the onset of inflammatory disease [133]. Inflammatory diseases such as asthma, allergies, rheumatoid arthritis and inflammatory bowel disease, among others, require therapeutic interventions to prevent tissue damage and organ dysfunction. Despite notable advances in anti-inflammatory therapies, long term use of currently available steroidal and non-steroidal anti-inflammatory drugs (NSAIDS) are limited by drug-induced toxicities [134]. Thus, there is currently a large unmet medical need for development of new and more potent anti-inflammatory agents with unique or novel mechanisms of action that show high synergistic efficiency with current agents in order to minimize any associated toxicities.

Amino acid deprivation (AAD) is emerging as a key natural mechanism that the body uses to regulate immune responses and to suppress immune cell activation and function. Immunoregulatory enzymes, such as arginase and indoleamine 2,3-dioxygenase (IDO) exert localized immunomodulatory effects on immune cells through catabolizing the amino acids arginine and tryptophan, respectively, to create a low amino acid environment [31,135]. Immune cells are exquisitely sensitive to amino acid deprivation compared to other cells in the body. Indeed, IDO is important in the regulation of many different types of immune responses, such as preventing semi-allogeneic fetus rejection [51], and in the pathological conditions including neoplasia [60,61], chronic infection [31], asthma [58], and autoimmune diseases [53].
Moreover, in a model of allergic airway inflammation, the glucocorticoid dexamethasone was shown to exert IDO-dependent protection, indicating AAD might represent an important mechanism of action that corticosteroids use to elicit their anti-inflammatory effect [59].

It has been suggested that the stress-responsive kinase general control non-derepressible 2 (GCN2) pathway allows T-cells to sense and respond to low amino acid conditions generated by IDO [78]. Mechanistically, amino acid depletion can cause a rise in the levels of uncharged transfer RNAs (tRNAs) in cells that activates the regulatory domain of GCN2, triggers it kinase activity and initiates the downstream signaling resulting in phosphorylation of eukaryotic initiation factor 2α (eIF2α) [119]. Phosphorylation of eIF2α leads to inhibition of general protein synthesis while simultaneously stimulating synthesis of specific stress response proteins of the integrated stress response (ISR) [84,120]. Amino acid deprivation also antagonizes a second well characterized highly conserved nutrient sensing pathway: mammalian target of rapamycin (mTOR). In response to energy or nutrient deprivation, the inhibition of TOR slows the rate of protein translation, ribosomal biogenesis, and cell division – the sum of effects is a halt or reduction is cell growth and proliferation [90,136]. Therefore, creating an amino acid depleted environment may regulate synthesis of inflammatory mediators and dampen an inflammatory response.

Previous findings from our group suggest borrelidin, a threonyl tRNA synthetase inhibitor, is a potent suppressor of T-cell activation and proliferation. Aminoacyl tRNA synthetase (aaRS) inhibitors, such as borrelidin, block the aminoacylation of specific amino acids onto their cognate tRNAs creating an amino acid depleted environment. In addition to our work with borrelidin, aaRS inhibitors have been shown to decrease the proliferation of immune cells in a mixed lymphocyte reaction [107] and suppress p70S6K activity, a well-known
downstream marker of mTOR activity [113], demonstrating aaRS inhibitors may hold utility as immune regulators.

Because amino acid deprivation is emerging as a key natural mechanism used to regulate immune responses, we decided to examine borrelidin’s potential to inhibit lipopolysaccharide (LPS)-induced cytokines production from macrophages. Macrophages are considered to play essential roles in inflammation. When activated by endotoxin, macrophages produce inflammatory cytokines which in turn activate other macrophages and other nearby cells to promote gene induction. Therefore regulation of cytokine expression in macrophages is an important strategy for controlling inflammatory responses. In this study, we provide evidence that borrelidin can suppress LPS-induced cytokine production at the level of protein synthesis.

3.2 Materials and methods

3.2.1 Cell isolation and culture

Spleen cell suspensions from BALB/c, CD1, or C57BL/6 mice were collected, and NH₄Cl was added to lyse erythrocytes (Red Blood Cell Lysing Buffer Hybri-Max, Sigma Aldrich Inc., St. Louis, MO). Splenocytes were resuspended in complete growth medium RPMI 1640 (Hyclone, Ottawa, ON), containing 50 U/ml penicillin and 50 µg/ml streptomycin (Gibco, Carlsbad, CA), 50 µM beta-mercaptoethanol (Sigma-Aldrich, Inc., Saint Louis, MO), 1 mM sodium pyruvate (Gibco, Carlsbad, CA), and 2 mM L-glutamine (Gibco, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS) (Hyclone, Ottawa, ON) and challenged with 0.1 µg/ml LPS (Sigma Chemical Co. St. Louise, MO) in a titration of borrelidin (0 nM - 306 nM) (Axxora Life Sciences Inc., San Diego, CA) or ochratoxin A (0 - 40 µM) (Sigma Chemical Co. St. Louise, MO) in a 96 well plate at a cell density of 1x10⁶ cells/well. 3 hours later, cells
were pelleted and the supernatants were collected and frozen at -20°C to be later assessed by either TNFα ELISA or a cytokine multiplex immunoassay. The phosphorylation levels of eIF2α and rpS6 were analyzed by intracellular staining and flow cytometry. Purified splenic macrophages were obtained from splenocytes by plastic adherence as previously described [137]. Briefly, splenocytes were plated in a 10cm² plate for 2 days at 37°C in complete growth media supplemented with 5 ng/ml mCSF-1 (Sigma Aldrich Inc., St. Louis, MO), after which nonadherent cells were gently removed by washing the dish with culture medium three times. For the measurement of TNFα mRNA and protein intracellular levels, splenic macrophages were challenged with 0.1 μg/ml LPS in the presence (20.4 nM) or absence (0 nM) of borrelidin for 3 hours. 1 μL of BD GolgiPlug was added per ml of culture medium for 1 hour prior to the LPS and borrelidin addition when assessing the intracellular TNFα expression levels. After 3 hour LPS stimulation cell were washed twice and lysed in TRIzol for RNA quantification or lysed in an ice-cold non-reducing lysis buffer for determination of intracellular TNFα levels.

3.2.2 Preparation of borrelidin

Borrelidin was purchased from Axxora Life Sciences Inc. (San Diego, CA) and reconstituted in dimethyl sulfoxide (DMSO) (Sigma Aldrich Inc., St. Louis, MO) at 1mg/ml and stored at -20°C until use. Borrelidin was diluted in culture medium prior to use. DMSO without borrelidin was used as a control.
3.2.3 Measurement of TNFα

The concentrations of TNFα in splenocyte supernatants or splenic macrophage lysates were assessed using commercially available OptEIA Mouse TNF ELISA Set according to the manufacturer’s instructions (BD Biosciences, II, Mississauga, ON).

3.2.4 Cytokine multiplex immunoassay methods

Splenocyte supernatants were thawed and analyzed with the multiplex cytokine detection system. The cytokine expression profile in each sample was determined using a cocktail of antibody-coated beads concurrently measuring the levels of 23 different cytokines (Bio-Plex mouse cytokine 23-plex immunoassay, Bio-Rad Laboratories, Hercules, CA, USA). This technology allowed for measurement of multiple analytes in a single 50 μl sample. The analysis was performed following the manufacturer's instructions and results were generated using the Bio-Plex 200 system and software.

3.2.5 RNA isolation

Total RNA extraction of primary splenic macrophages was carried out after harvesting using TRIzol (Gibco Life Technologies, Carlsbad USA) according to the manufacturer's instructions. RNA concentration was measured by reading the absorbance at 260/280 nm using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).
3.2.6 Quantitative PCR

Total RNA (1 µg) was reverse transcribed using the Superscript first-strand synthesis system for reverse transcription-PCR as described in the manual (Invitrogen). Synthesized cDNA (5 µl) was combined in a PCR with TNFα specific primers (forward, 5'-TTGTTGCTCCTCTTTTGCT-3'; and reverse, 5'-TGGTCACCAAAATCAGCGTTA-3') or CHOP specific primers (forward, 5'-CTGCACCAAGCATGAACAGT-3'; and reverse, 5'-CTACCCTCACTCCCTCCTC -3'). TNFα and CHOP mRNA levels were normalized to the level of Ubiquitin C and RPL32, respectively, by using Ubiquitin C specific primers (forward, 5'-CTAAGACACCTCCCCCATCA -3'; and reverse, 5'-AGCCCAGTGTTACCACCAAG -3') and RPL32 specific primers (forward, 5'-GCCAGGAGACGACAAAATC-3'; and reverse, 5'-AATCCTCTTGCCCTGATCCT-3') and Platinum Sybr green PCR supermix (Invitrogen). Amplification was performed on an Applied Biosystems 7900HT fast real-time PCR system. Ubiquitin C was validated as an endogenous control for the TNFα-specific primers and RPL32 was validated as an endogenous control for the CHOP-specific primers and dissociation curves resulted in a single peak of PCR amplification.

3.2.7 Cell extract preparation

For measurement of intracellular TNFα levels, splenic macrophages were stimulated as described above. Cells were lysed in an ice-cold non-reducing lysis buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% Glycerol, 0.5 mM phenylmethyl-sulfonyl fluoride, 3.3 µg/ml aprotinin) for 30 min at 4°C. Cell lysates were centrifuged 13,000 g for 20 min at 4°C. Supernatants were collected and frozen at -20°C to be later assessed by TNFα ELISA.
3.2.8 Analysis of intracellular phospho-eIF2α and phospho-S6

Activated splenocytes were first stained on ice for 1 hour with a PE-conjugated anti-CD11b antibody (BD, Mississauga, ON). For intracellular phospho-eIF2α detection, cells were then fixed using BD Cytofix, following the manufacturer’s instructions, and permeabilized in 0.2% triton-X in PBS for 20 minutes at room temperature. After permeabilization, cells were washed once in Tris-buffered saline (TPBS) (PBS + 0.05% Tween 20) and blocked with 5% normal goat serum (NGS) (Vector Labs, Burlington, ON) in TPBS for 20 minutes at room temperature. Cells were then incubated at 37°C for 1 hour with a 1:100 final dilution of anti phospho-eIF2α (Ser 51) antibody (Cell Signaling Technology, Danvers, MA) in 1% bovine serum albumin (BSA) (Invitrogen, Grand Island, NY)/1%NGS in TPBS. The secondary antibody was an APC-conjugated anti-rabbit IgG (H+L) (Jackson ImmunoResearch West Grove, PA). For intracellular phospho-rpS6 detection, cells were then fixed and permeabilized by BD Cytofix/Cytoperm (BD, Mississauga, ON) for 20 min on ice. The alexa488-conjugated anti-phospho-rpS6 (Ser235/236) (Cell Signaling Inc., Danvers, MA, USA) was diluted 1:10 in BD Perm/Wash (BD, Mississauga, ON) and incubated overnight at 4°C. Cells were analyzed using Becton Dickinson FACS Canto II flow cytometer and FlowJo software. The analysis of the phospho-eIF2α and phospho-rpS6 populations was based on CD11b-positive gated population. 10,000 events were collected for each sample based on the CD11b-positive gated population. Gates for CD11b, phospho- eIF2α, and phospho-rpS6 were set based on the unstimulated single stained controls.
3.2.9 Statistical analysis

Statistical analyses were performed using Student’s t test. Significance was assessed at the 95% confidence level.

3.3 Results

3.3.1 Borrelidin suppresses cytokine production in LPS–stimulated splenocytes

First we asked whether borrelidin could attenuate activation of an inflammatory response. To do this, we added increasing concentrations of borrelidin (0 - 306 nM) to primary splenocytes stimulated with LPS (0.1 µg/ml) for 3 hours and then collected conditioned media to assay for cytokine levels. In the absence of borrelidin, activated splenocytes produced a substantial amount of tumor necrosis factor alpha (TNFα) in response to LPS (Figure 3.1a). When borrelidin was added to splenocytes and challenged with LPS, TNFα was suppressed in a dose-dependent manner. Concentrations of borrelidin over 10 nM were able to significantly suppress the level of TNFα produced in response to LPS with a half maximal inhibitory concentration (IC_{50}) of 22 nM (Figure 3.1A). There was no significant difference in the viability between borrelidin (300 nM) treated and untreated splenocytes at the end of the 3 hour LPS challenge, indicating borrelidin suppressed TNFα production without affecting cell viability (Figure 3.1B). No TNFα was detected in the cell culture supernatant of non-LPS stimulated cells.

Since the inflammatory response involves a number of cytokines, we used a multiplex system to evaluate borrelidin’s suppressive effects on a panel of inflammatory cytokines. Primary splenocytes were treated with borrelidin (0 - 306 nM) and challenged with 0.1 µg/ml LPS for 3 hours. As shown in Figure 3.2, whilst LPS-induced production of interleukin-10 (IL-
10), IL-12, RANTES, macrophage inflammatory protein 1α (MIP-1α), and MIP-1β were significantly upregulated by LPS alone, production of these cytokines were suppressed dose dependently by borrelidin (Figure 3.2).

Figure 3.1 Inhibition of TNFα induction by borrelidin
Splenocytes were treated with (1.3-300 nM) or without (0 nM) borrelidin and stimulated for 3 hours with LPS (0.1 µg/ml). At 3 hours, conditioned media was harvested and the amount of TNFα produced was measured by ELISA (A). At the same time, cell viability was assessed with trypan blue (B). The means and standard deviations of three experiments are presented. Statistical significance (p < 0.05*) was determined by comparing the condition with the untreated stimulated condition (0 nM borrelidin, 0.1 µg/ml LPS stimulated). ND: too low to detect
Figure 3.2 Borrelidin inhibits LPS induced cytokine release in primary splenocytes
Splenocytes were treated with (1.3 - 300 nM) or without (0 nM) borrelidin and stimulated for 3 hours with LPS (0.1 μg/ml). At 3 hours, conditioned media was harvested and analyzed using BioRad’s Bio-Plex Pro Mouse Cytokine 23-plex Assay. The means and standard deviations of three experiments are presented. Statistical significance (p < 0.05*) was determined by comparing the condition with the untreated stimulated condition (0 nM borrelidin, 0.1 μg/ml LPS stimulated) ND: too low to detect

3.3.2 Borrelidin does not inhibit mRNA expression of TNFα

To better understand how borrelidin regulates cytokine expression, we first looked at the transcription of TNFα mRNA in borrelidin treated primary splenic macrophages stimulated with 0.1 μg/ml LPS. Inhibition of mRNA transcription by interfering with DNA binding of transcription factors at proinflammatory gene promoters [138,139] and repression or sequestration of transcriptional regulators through protein-protein binding to prevent recruitment of key co-activators, chromatin modifiers, or components of basal transcriptional machinery
[140-142] are just a few mechanisms used by current anti-inflammatory therapeutics to regulate cytokine expression levels. As shown in Figure 3.3, treatment of LPS-stimulated splenic macrophages with 20.4 nM borrelidin for 3 hours did not inhibit mRNA expression levels of TNFα as assessed by RT-PCR analysis (Figure 3.3A). Rather, the addition of borrelidin increased the levels of TNFα mRNA by 3.7 fold as compared to the LPS-stimulated splenic macrophages in the absence of borrelidin (Figure 3.3A). We also showed that 3 hours of 0.1 μg/ml LPS stimulation of primary splenic macrophages with 20.4 nM borrelidin induced a 5 fold increase in CHOP mRNA levels (Figure 3.3B). This suggests borrelidin is activating the GCN2 pathway, as CHOP is a well know and characterized marker for GCN2 activation [108], and that borrelidin is not inhibiting the expression of TNFα at the transcriptional level.

**Figure 3.3 Borrelidin does not inhibit TNFα mRNA transcription in splenic macrophages**

Relative quantities (Rq) of TNFα and CHOP from splenic macrophages are shown by qPCR following a 3 hour stimulation with 0.1 μg/ml LPS. Ubiquitin C and Ribosomal protein L32 (RPL32) were used as endogenous controls, respectively. RQ values were calculated from ∆Ct values. Statistical significance (ρ < 0.05*) was determined by comparing the condition with the untreated unstimulated condition (0 nM borrelidin, 0 μg/ml LPS stimulated)
3.3.3 Borrelidin does not inhibit secretion of TNFα

Having demonstrated that borrelidin was not impeding TNFα mRNA transcription but was inhibiting production of TNFα in the conditioned media, we next examined if borrelidin was suppressing TNFα through dysregulation of cytokine secretion. Primary macrophages were treated for 1 hour with BD GolgiPlug, a protein transport inhibitor containing brefeldin A, prior to LPS stimulation. 3 hours following 0.1 μg/ml LPS stimulation, condition media and cell lysate were assayed for TNFα levels using ELISA. BD GolgiPlug was able to completely block release of TNFα from the LPS stimulated splenic macrophages (Figure 3.4A). LPS induced an increase in the intracellular levels of TNFα, which was attenuated 3.8 fold by 20.4 nM borrelidin (Figure 3.4B). Since the total intracellular levels of TNFα are significantly lower upon 20.4 nM borrelidin treatment regardless of whether proteins can be secreted or not, it suggests TNFα protein levels in activated splenic macrophages rely on protein synthesis and not transcription of mRNA or secretion of the cytokine.
3.3.4 Borrelidin signals through the GCN2 and mTOR pathways in primary splenic macrophages

Recent studies suggest that the stress-responsive kinase general control non-derepressible 2 (GCN2) signaling pathway enables T-cells to sense and respond to low amino acid conditions generated by IDO [78]. We therefore asked whether macrophages also used the GCN2 pathway to sense and respond to low amino conditions created by borrelidin. To address this question, we examined the phosphorylation levels of eIF2α, the direct downstream substrate of GCN2, using intracellular immunofluorescent staining and flow cytometry. As shown in Figure 3.5A, intracellular staining revealed borrelidin in LPS-stimulated CD11b+ splenocytes promotes phosphorylation of eIF2α in a dose dependent manner (Figure 3.5A). The mTOR signaling pathway was also assessed by examining the phosphorylation states of rpS6, a widely used
marker to monitor the activation state of mTOR [125]. Borrelidin was able to dampen rpS6 phosphorylation in CD11b+ splenocytes (Figure 3.5B). For both eIF2α and rpS6 staining, stimulation in tryptophan deficient media was used as a control for amino acid deprivation induced signaling.

Figure 3.5 Borrelidin induces eIF2α phosphorylation and inhibits phosphorylation of ribosomal protein S6 in CD11b positive splenocytes
Splenocytes were stimulated with 0.1 µg/ml LPS in the presence of the indicated concentrations of borrelidin. After 3 hours, the levels of intracellular eIF2α (A) and phosphorylated rpS6 (B) were monitored using intracellular staining and flow cytometry and the average mean fluorescence intensities (MFI) were collected from CD11b-positive gated populations. The ratio of MFI for each treatment was compared against the 0 nM borrelidin, 0.1 µg/ml stimulated sample to determine the fold change in phosphorylation. Statistical significance (p < 0.05*) was determined by comparing the condition with the untreated stimulated condition (0nM borrelidin, 0.1 µg/ml LPS stimulated)
3.3.5 Ochratoxin A suppresses cytokine production in LPS – stimulated splenocytes

As borrelidin was able to potently inhibit TNFα production from LPS-stimulated splenocytes, we examined whether a second aaRS inhibitor, ochratoxin A, would also be able to attenuate TNFα production. Thus, we monitored LPS-induced TNFα levels in splenocytes treated with ochratoxin A. Indeed, ochratoxin A was also able to inhibit the production of TNFα in a dose dependent manner — at a 1000 fold higher concentration as borrelidin (Figure 3.6).

**Figure 3.6 Inhibition of TNFα production by ochratoxin A**

Splenocytes were treated with (5 - 40 μM) or without (0 μM) ochratoxin A and stimulated for 3 hours with LPS (0.1 μg/ml). At 3 hours, condition media was harvested and the amount of TNFα produced was measured by ELISA. Statistical significance (p < 0.05*) was determined by comparing the condition with the untreated stimulated condition (0nM borrelidin, 0.1 μg/ml LPS stimulated)
3.4 Discussion

Macrophages are considered to play an essential role in inflammation. When activated by endotoxins, macrophages produce inflammatory cytokines, which in turn activate other macrophages and other nearby cells to promote gene induction of further inflammatory mediators. When such responses become dysregulated or chronically activated, however, they contribute to the development of inflammatory disease. Hence regulation of cytokine expression in macrophages is an important strategy for controlling inflammatory responses.

Amino acid depletion might represent an important mechanism of action of anti-inflammatory corticosteroids and a new therapeutic approach to several pathologic conditions. Glucocorticoids, such as dexamethasone, are shown to exert IDO-dependent protection in a model of allergic airway inflammation [59] and in asthmatic patients [143]. Following one week of bronchial colonization of *A. fumigates* in T\(_H\)2-primed mice, the number of eosinophils, the amount of mucin secretion in the bronchoalveolar lavage fluid, the amount of circulating IgE antibody, and the amount of lung hydroxyproline – all indicative of *A. fumigates* respiratory allergy [144] – were decreased in dexamethasone-treated mice [59]. These decreases were not seen in untreated mice nor in mice co-treated with dexamethasone and the IDO inhibitor, 1-MT, suggesting tryptophan catabolism may be an important mechanism of action for the anti-inflammatory corticosteroid, dexamethasone. Therefore, in the present study we aimed to explore the anti-inflammatory potential of borrelidin, a ThrRS inhibitor.

Our first step was to examine a key function of monocytes and macrophages – the secretion of cytokines in response to an inflammatory stimulus such as LPS. We tested the effects of borrelidin at varying concentrations in primary splenocytes stimulated with LPS. Our results demonstrated that borrelidin was able to suppress TNF\(\alpha\) production from primary
splenocytes in a dose-dependent manner without effecting cell viability, since cell viability at the end of the stimulation period did not differ with culture conditions. Similar results were obtained for IL-10, IL-12, RANTES, MIP-1α and MIP-1β when examining a panel of cytokines using a multiplex approach. Inhibition of cytokines, particularly TNFα, has been successful in several clinical trials for the treatment of rheumatoid arthritis [145], psoriatic arthritis [146], Crohn’s disease [147,148], ulcerative colitis [149], refractory asthma [150], and ankylosing spondylitis [151]. Since borrelidin is able of inhibit multiple inflammatory cytokines, it may hold utility as an anti-inflammatory therapy, possibly by working synergistically with current anti-TNFα therapies to minimize their unwanted side effects.

Unlike current anti-TNFα therapies that bind secreted TNFα, we found borrelidin prevented the production of TNFα without disrupting TNFα mRNA expression or secretion of TNFα protein. These results were expected, as borrelidin has been well characterized as a non-competitive inhibitor of ThrRS, preventing the aminoacylation of threonine [114,152]. Interestingly, we observed higher levels of TNFα mRNA with the addition of borrelidin upon LPS stimulation. This could be a result of stress granule formation. In response to stress, RNA-binding proteins, TIA-1 and TIAR, co-localize with AU-rich elements in the 3’-untranslated regions found in many inflammatory mediators. Once associated, TIA-1 and TIAR assemble into stress granules that harbor the non-essential untranslated mRNA until the stress is over [153]. Interestingly, phosphorylation of eIF2α is sufficient to induce translational arrest and stress granule assembly [154]. Therefore, the increase of TNFα mRNA in borrelidin treated LPS stimulated splenic macrophages could be due to the cells “storing” the mRNA in stress granules until the amino acid depleted stress is over.

The GCN2 kinase pathway has been recently suggested as an intracellular signaling pathway in T cells that mediates key biological effects of IDO [78]. In this study, by detecting
the phosphorylated levels of eIF2α, the direct downstream substrate of GCN2, we have shown that there is a positive correlation between increasing dose of borrelidin and eIF2α phosphorylation in CD11b+ splenocytes. There was also a significant increase of eIF2α phosphorylation in the absence of any LPS stimulus, suggesting the CD11b+ splenocytes require stimulation in cell culture in order to be maintained in cell culture. We have also shown under the same experimental conditions that the level of phosphorylated rpS6 decreases with increasing borrelidin concentration. Our results suggest borrelidin is activating the GCN2 pathway and inhibiting the mTOR pathway in CD11b+ cells and this may be one of the underlying mechanisms by which borrelidin is eliciting its anti-inflammatory activity.

To expand our understanding on aaRS and their ability to suppress cytokine release from splenocytes, we also looked at the PheRS inhibitor, ochratoxin A (OTA). OTA is a naturally occurring mycotoxin produced by several strains of *Aspergillus* and *Penicillium* which inhibits protein synthesis by competing with phenylalanine for phenylanyl-tRNA synthetase [155]. We found OTA was able to suppress TNFα production dose-dependently, suggesting aaRS inhibitors as a class may exhibit anti-inflammatory properties and should be further examined in pursuit of novel anti-inflammatory agents.

In summary, we have demonstrated that borrelidin inhibits TNFα protein expression without inhibiting its mRNA expression or secretion from primary splenic macrophages. This may be important in the understanding of a novel mechanism for borrelidin. Furthermore, our data extends our understanding of the molecular mechanisms underlying the biological activity of borrelidin. However, further study into the precise mechanism by which borrelidin inhibits cytokine release is needed through further study with the GCN2 and mTOR signaling pathways.
CHAPTER 4. Discussion

4.1 General discussion and conclusions

Abnormal immune responses require clinical intervention to prevent tissue damage and organ dysfunction. Unfortunately, many of these clinical interventions have undesired side effects; therefore, development of novel therapeutic agents with different mechanisms of action would be immensely beneficial for the treatment of autoimmune and inflammatory diseases.

Mounting evidence in *in vitro* and *in vivo* studies implicate amino acid depletion may be one natural mechanism by which the body uses to elicit localized immunosuppression. A well-studied example by which immunosuppression is achieved is through expression of the tryptophan catabolizing immunoregulatory enzyme IDO. IDO expression in trophoblast cell of the placenta creates a low tryptophan zone around the semi-allogeneic fetus which acts as a protective barrier against the maternal immune system [51]. In addition to the studies that suggest IDO plays an important role in the regulation of many different types of immune responses [53-63], other amino acid catabolising enzymes [67-70], as well as the abundance of specific amino acids [71-73, 75-77] have been linked with immunoregulatory effects. Strategies to induce an amino acid depleted environment may be advantageous in inhibiting unwanted immune reactions during transplantation, chronic inflammatory and autoimmune diseases.

One way to induce an amino acid depleted environment is through inhibiting aminoacyl-tRNA synthetases (aaRSs). Inhibitors of aaRSs limit the cell’s availability to specific amino acids and thereby create an environment with low amino acid levels. Because of increasing compelling evidence that amino acid depletion can induce immunosuppression, we believe aaRS inhibitors hold potential as immunosuppressive agents. In support of our hypothesis, aaRS
inhibitors have been shown to decrease the proliferation of immune cells in a mixed lymphocyte reaction [107] and suppress p70^{S6K} activity [113]. Therefore, as part of this thesis, we aimed as to further elucidate whether borrelidin, a ThrRS inhibitor, elicits an immunosuppressive effect on primary immune cells.

Borrelidin may be a key immune regulator because of two key functions. Because borrelidin inhibits ThrRS, there is automatically an increase in the levels of uncharged tRNA within the cell, which directly activates the GCN2 pathway. Secondly, because borrelidin prevents threonine from coupling with its tRNA, free threonine within the cell gets rapidly catabolised and utilized in other cellular processes [112]. Therefore, we believe borrelidin works at two levels: increasing the tRNA levels to active GCN2 and also creating a threonine depleted environment, inhibiting mTOR.

4.1.1 Borrelidin as an immunosuppressive agent

To address this question, as described in Chapter 2, we performed a series of experiments in which we show that borrelidin is able to potently inhibit the proliferation, activation and cytokine production in T-cells. These studies were important as our first step in understanding if borrelidin could be immunosuppressive, as T-cells alone are both necessary and sufficient to drive acute graft rejection [156]. Borrelidin was able to very efficiently inhibit the proliferation of T-cells in a mixed leukocyte reaction (MLR) with an IC_{50} of 3.3 nM. In fact, when compared against other immune cell types: macrophages, mast cells, and B-cells, borrelidin was able to more effectively inhibit T-cells. Furthermore, we showed primary fibroblasts from foreskin and HACAT cells exhibit an approximate 300-fold higher tolerance to borrelidin’s suppression.
Selectivity for immune cells is an essential component to consider when it comes to the development of new immunosuppressive agents.

It is not surprising that regulation of T-cell activity can be achieved by limiting nutrient availability. Generating an immune response is a considerable bioenergetic challenge. T-cells must be able to shift from a resting, quiescent phenotype to a highly active metabolic state within hours of stimulation. Rapid rates of growth, proliferation, and activation of effector function depends on the ability of T-cells to increase their biomass, replicate their genomes, increase rates of protein synthesis, and increase ATP production. In order to meet the energy demands and respond to pathogens, T-cells must also actively acquire metabolic substrates from their extracellular environment at a sufficient rate to meet the demands of proliferation [157,158]. Analogous to many neoplastic and transformed cells, proliferating T-cells have been shown to be hypersensitive to pharmacological manipulation of particular metabolic control pathways. Drugs that interfere with energy metabolism are shown to be potent immunotherapeutics for patients with autoimmune or infectious disease or for those who are undergoing solid-organ transplantation. This is exemplified by rapamycin which inhibits protein synthesis by suppressing mTOR [159].

AAD is emerging as a natural mechanism for mediating immunosuppression. When amino acid levels become limiting, cells activate a bioenergetics and metabolic conservation mode called the integrated stress response (ISR). When cells enter this quiescent state, translation of non-essential mRNAs is inhibited, diverting energy and resources to maintain cell survival. Therefore, it stands to reason activating IRS and/or inhibiting mTOR, such as in the case of borrelidin, can lead to rapid impairment of growth and proliferation by encumbering the high energetic demands of activated T-cells. The high metabolic demands may be one
contributing factor for why we observed that T-cells exhibit a higher level of sensitivity to borrelidin when compared with other primary immune cells and cell lines.

From recent findings in our lab, we speculate the expression of IMPACT, a known GCN2 inhibitor which is highly and constitutively expressed in skin but absence in immune cells [131,132], may be protecting non-immune cells from borrelidin-induced GCN2 activation. It has been shown that IMPACT overexpression in amino acid deprived conditions leads to inhibition of GCN2 and abolishes expression of CHOP, a well-known downstream marker of GCN2 activity [132,160]. Moreover, IDO-induced suppressive as well as apoptotic effect was demonstrated in IMPACT knocked-down fibroblasts co-cultured with IDO-expressing fibroblasts [131]. This suggests IMPACT expression levels may be a contributing factor to why HACAT and fibroblasts are more resistant to borrelidin induced suppression of proliferation.

Activation of the GCN2 pathway as detected by eIF2α phosphorylation was seen upon borrelidin treatment of T-cells. This is an important finding as activation of GCN2 is a known pathway which responds to the low amino acid conditions created by IDO to mediate proliferative arrest and anergy induction [78]. Furthermore, mTOR activity was significantly lower in borrelidin treated T-cells as monitored by phosphorylated rpS6 levels. Congruent with our results, suppression of tRNA aminoacylation by aaRS inhibitors is able to inhibit p70^{S6K}, the kinase responsible for phosphorylating rpS6 [113]. Identifying borrelidin is signaling through these nutrient sensing pathways in CD3+ T-cells which have already been associated as intracellular mechanisms by which amino acid depletions elicits immunosuppression, supports that borrelidin may be regulating T-cell proliferation and function in a similar capacity as IDO-induced immunosuppression.
Although the precise mechanism by which mTORC1 responds to amino acids is unclear, it is thought that the accumulation of amino acids inside lysosomes activate Rag GTPases via the v-ATPases-Ragulator interaction, causing Rags to recruit mTORC1 to the lysosome and become activated by Rheb [105]. As such, in the absence of amino acids in the lysosome or over expression of PAT1, a proton-coupled amino acid transporter specifically localized to the lysosome which exports amino acids out of the lysosomal lumen, mTORC1 signaling is attenuated [105]. When aaRSs are inhibited, such as in the case of borrelidin, specific amino acids are unable to attach with their cognate tRNA. These free amino acids within the cell get rapidly catabolized and utilized in other cellular processes. Therefore, one way borrelidin may be inhibiting mTOR, may be though decreasing the levels of threonine within the lysosome.

The findings from this aim also support the rationale that amino acid depletion alone can induce immune suppression. It is theorized that one way tryptophan catabolism by IDO facilitates immunosuppression is through a buildup of downstream metabolites of tryptophan that act to suppress immune cells proliferation and increase their susceptibility to apoptosis. Considerable amounts of evidence support this theory [161-167], including in vivo studies which show tryptophan metabolites alone, such as 3-hydroxyanthranillic acid (3-HAA), induce the generation of Tregs, inhibit T_H1 and T_H17 cells, and mitigate experimental autoimmune encephalomyelitis (EAE) [168]. Our results, on the other hand, support the idea that IDO catabolism of tryptophan suppresses T-cell proliferation by reducing the abundance of this critical amino acid, as borrelidin decreases the intracellular levels of theryonyl-tRNA. This second theory was first suggested by the observation that some effects of IDO on T-cells are reversed by the addition of excess tryptophan [78,169]. Similarly, our results indicate the ability of borrelidin to suppress T-cell proliferation can be reversed by an excess of threonine. In the context of IDO, it is most likely a combination of tryptophan depletion and tryptophan
metabolites that contributes to IDO – mediated immunosuppression. However, since borrelidin is currently not known to induce an increase in tryptophan metabolites, our results as well as studies examining the direct effect of amino acid levels on immune regulation [72,73,75-77], support that the depletion of a specific amino acid alone can regulate immune cells.

These finding cumulatively suggest for the first time that borrelidin is able to potently suppress T-cell proliferation, activation and cytokine release and that borrelidin is able to activate the GCN2 and inhibit the mTOR pathways. The findings of this study help us to have a better understanding of the role of aaRS inhibitors have in suppressing immune function. They may also lead to the potential application of borrelidin as a therapeutic agent for the treatment of immunological diseases or in transplantation. Borrelidin may work well as a single agent therapy, or may work in combination with existing approved therapeutics, minimizing the dose of current therapies and hopefully minimize the unwanted side effects.

4.1.2 Borrelidin as an anti-inflammatory agent

Many of the studies into amino acid depletion as a mechanism of immune regulation have been performed studying T-cells. Although T-cells are both necessary and sufficient for virtually all rejection of allogeneic organs and tissues, advances in the understanding in the innate immune response has highlighted the critical role of the innate immune system in shaping the adaptive response [170]. Therefore, we wanted to investigate whether borrelidin could elicit an anti-inflammatory effect in addition to being immunosuppressive. We believe borrelidin may be an effective anti-inflammatory agent from studies that suggest that glucocorticoid exert an IDO-dependent in models of allergic airway inflammation [59] and in asthmatic patients [143]. These studies, along with others examining the suppressive effects of IDO on T_{H}2 cells in
inflammation models [171], suggests tryptophan catabolism may be one way to regulate inflammation.

As discussed in Chapter 3, borrelidin was able to inhibit LPS-induced cytokine production from splenocytes without effecting cell viability, suggesting the potential efficacy of this small molecule in managing the production of cytokines during an inflammatory response. Pharmaceutical companies have been utilizing cytokine inhibition as a mechanism of therapeutic intervention in a variety of inflammatory diseases: most notably is the inhibition of TNFα through circulating monoclonal antibodies or receptor fusion proteins [145-151]. Since borrelidin is able of inhibit multiple inflammatory cytokines, it may hold utility as an anti-inflammatory therapy, possibly by working synergistically with current anti-TNFα therapies to minimize their unwanted side effects. Moreover, the ability of borrelidin to suppress cytokine production will ultimately influence the development and phenotype of both innate and adaptive immune cells, potentially preventing or attenuating harmful activities [172].

An important finding of Chapter 3 was identifying that borrelidin was inducing activation of the GCN2 pathway and inhibition of the mTOR pathway. These pathways have been linked with amino acid deprivation and inhibition of T-cell proliferation and activation and our data in Chapter 2 suggests borrelidin may be utilizing the pathways in T-cells. Yet, in Chapter 3, we were also able to identify that borrelidin induced amino acid depletion is signaling through GCN2 and mTOR in CD11b+ cells. Since one key effector function of both of these pathways is to regulate protein synthesis by regulating translation machinery, it stands to reason that activation and inhibition of GCN2 and mTOR pathways, respectively, leads to a decrease in protein synthesis. Therefore, we propose the decrease in cytokines seen with increasing borrelidin concentrations is due to activating GCN2 and inhibiting mTOR to cause decrease in protein synthesis of cytokines. Further results of Chapter 3 support this theory, as we identified
borrelidin is neither inhibiting mRNA transcription nor secretion of TNFα. Our results indicate amino acid depletion in macrophages may be a mechanism of controlling inflammatory responses.

These results suggest borrelidin holds utility as an anti-inflammatory agent. The benefit of using an agent that targets multiple cell types is that it would be able to control early immune responses and shape further adaptive immune responses.

4.1.3 General conclusions

The studies in this thesis support the notion that borrelidin, a ThrRS inhibitor, has immunosuppressive and anti-inflammatory activities. These findings help us to better understand the role of aaRS inhibitors in regulating immune function. We speculate that other aaRSs inhibitors may show selective immunosuppressive effects as well. These small molecule inhibitors may also have potential use as novel therapies in immune-related diseases and transplantation. Small molecule inhibitors of aaRS are therapeutically advantageous over larger enzyme proteins as they are more stable, less immunogenic, and less allergenic [173,174]. Borrelidin may work well as a single agent therapy, or may work in combination with existing approved therapeutics, minimizing the dose of current therapies and hopefully minimizing unwanted side effects.

In summary, in this thesis we were able to: demonstrate borrelidin potently and more effectively inhibits T-cell activation, proliferation and cytokine release; determine borrelidin is able to prevent cytokine release upon LPS stimulation without effecting cell viability; and elucidate that the GCN2 and mTOR signaling pathways in both T-cells and macrophages respond to the low amino acid conditions borrelidin creates.
4.2 Suggestions for future work

Although we believe that our findings support the potent immunosuppression activities of borrelidin, there is still a considerable amount of studies that needs to be performed in this area of research. The following are some of my suggestions of studies that can be done to further support and add to my findings:

1. In Chapter 2, our data indicates that very low concentrations of borrelidin are able to suppress the proliferation of T-cells. These results suggest the potential therapeutic application for immunological diseases and transplantation. Therefore, we suggest: determining the pharmacokinetic profile of borrelidin; examining borrelidin’s toxicology; and examining the *in vivo* efficacy of borrelidin in the treatment of models of autoimmunity and transplantation.

2. Our Chapter 2 results also indicate borrelidin may be a selective inhibitor of immune cells compared to non-immune cells. Further examination of the mechanism behind this selectivity is required. Therefore, we suggest: identifying if IMPACT protein levels contribute to the more resistant response of non-immune cells by knocking down IMPACT in fibroblasts and monitoring their response to borrelidin; monitoring apoptotic markers during proliferation assays to identify if borrelidin is killing T-cells.

3. In Chapter 3, we determined that borrelidin is able to inhibit TNFα production without effecting cell viability. However, we noted an increase in mRNA transcript levels. Further examination of what is happening to TNFα and other cytokine mRNA is needed. This can be done by examining stress granule formation and association of cytokine mRNA with TIA-1 and TIAR using fluorescence microscopy.
References


