THE USE OF A TEC KINASE INHIBITOR, IBRUTINIB, FOR THE
DEVELOPMENT OF IMMUNOTHERAPIES AGAINST CANCER AND
LEISHMANIASIS.

Dissertation

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ABSTRACT

Proteins belonging to the TEC family of kinases are critical for the function of immune cells. One of the kinases in this family, BTK (Bruton’s tyrosine kinase), is required for the development and functions of B cells. BTK also plays key roles during the pathogenesis of B cell malignancies. Hence, a therapeutic strategy against B cell malignancies involves the inhibition of BTK by small molecule inhibitors. In this respect, ibrutinib, an irreversible inhibitor of BTK, has shown promise in the treatment of B cell malignancies and has received FDA approval as a ‘Breakthrough Therapy Drug’ in 2013.

In addition to treating B cell malignancies, ibrutinib can potentially be used to target other immune cells that express BTK, such as dendritic cell (DCs). DCs, known as ‘sentinels of the immune system’, survey the body for the presence of ‘danger signals’ that result from infections and development of cancers. Following detection of such danger signals, DCs undergo maturation and increase the expression of surface activation markers, which include MHC-II and CD80. These activation markers serve to activate T cells. This process of DC-mediated T cell activation is critical for mounting a robust adaptive immune response to eliminate cancer cells or infectious agents. Previous studies showed that BTK deficiency enhances DC maturation, leading to higher expression of MHC-II and CD80. Further, BTK-deficient DCs promote higher levels of T cell activation compared to control DCs. Since ibrutinib inhibits BTK, we hypothesized that treatment of DCs with this inhibitor would enhance the expression of DC surface activation markers and promote DC-mediated T cell activation. Hence, we decided to evaluate the effect of ibrutinib on the maturation and activation of mouse bone marrow-derived DCs using an in vitro cell culture model. We isolated bone marrow cells from
mice and cultured these cells in the presence of a DC polarizing growth factor, GM-CSF (granulocyte-macrophage colony stimulating factor), for 7 days to generate DCs. On the first day of culture, DCs were exposed, or not, to the drug. As expected, we observed that inhibition of BTK enhanced the expression of MHC-II and CD80 in comparison to control untreated DCs. In a second set of experiments, treatment with the drug ibrutinib led to higher expression of MHC-II and CD80 following stimulation of DCs with an inflammatory stimulus called LPS (lipopolysaccharide). Upon LPS stimulation, ibrutinib-treated DCs also selectively enhanced the synthesis of some cytokines such as IFN-β and IL-10 while dampening the production of other cytokines such as IL-12. Further, LPS/ibrutinib-treated DCs promoted higher rates of T cell proliferation and production of T cell-derived cytokines compared to LPS only-treated DCs. In order to further analyze the mechanism underlying the increase in DC activation following ibrutinib treatment, we analyzed the expression of cytokines and nuclear proteins called ‘transcription factors’ that are involved in the development of DCs. We observed that treatment with the BTK inhibitor led to an increase in the induction of cytokines, IFN-α and IFN-β, as well as transcription factor, IRF-7. These proteins have previously been associated with enhanced maturation and activation of DCs. Taken together our study indicates that inhibition of BTK by ibrutinib promotes the development and activation status of DCs, which consequently respond more robustly to inflammatory stimuli and promote more efficient activation of T cells. These results provide a basis for exploring the impact of BTK inhibitors in the development of DC-based therapies against cancer and infectious diseases.
Since ibrutinib effectively inhibits the function of B cells, it could also be expanded for the treatment of infectious diseases involving B cells. B cells contribute to susceptibility to the parasitic, vector-borne disease cutaneous leishmaniasis (CL), which is characterized by localized lesions at the site of the vector sandfly bite. We hypothesized that inhibition of BTK using ibrutinib would promote resistance to CL. Mice footpads were infected with causative organism for CL, *Leishmania major*. From the first day of infection and throughout the course of the experiment, mice were administered vehicle (control) or ibrutinib *via* drinking water. We observed that mice treated with the inhibitor displayed slower progression of disease with smaller lesions and lower parasite burdens compared to vehicle-treated mice. These results indicate that inhibition of BTK curtails the progression of CL, thereby providing a rationale for the use of ibrutinib as a potential drug candidate for the treatment of cutaneous leishmaniasis.

In summary, both our studies demonstrate novel indications for BTK inhibitors, such as ibrutinib, for the development of DC-based therapies and for the treatment of leishmaniasis.
DEDICATION

Dedicated to my family, Amma, Appa, Sai and Shri
ACKNOWLEDGEMENTS

I believe that the process of becoming a scientist is one where we not only raise fundamental questions in science, but we also challenge our own worldviews. In this regard, I would like to sincerely thank my advisor, Dr. Abhay Satoskar, for providing me an intellectually-stimulating environment in his laboratory to become a rational thinker while giving me plentiful resources for my research as well as the training to become an independent scientist. I also value the numerous opportunities for professional development I received in Dr. Satoskar’s lab which were great stepping stones for a future career in research. I am also grateful to my committee members, Dr. Virginia Sanders, Dr. Stephanie Seveau and Dr. Pravin Kaumaya for their scientific inputs on my research projects and for immense support and mentoring during my tenure as a graduate student. I also thank the Chair of the Department of Microbiology, Dr. Michael Ibba, and the Chair of Graduate Studies Committee, Dr. Irina Artsimovitch for giving me an opportunity to be a part of this program. I would also like to thank all the members of the department especially Dr. Madhura Pradhan and Dr. Mette Ibba for their constant support as well as their valuable training during my time as a graduate teaching assistant.

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this project.

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http://dx.doi.org/10.1182/blood-2013-06-507947.

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**Peer-reviewed reviews and book chapters:**


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Major Field: Microbiology Graduate Program
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<th>Description</th>
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<tbody>
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<td>Abl</td>
<td>Abelson tyrosine kinase</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myelogenous leukemia</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BCR</td>
<td>B cell receptor</td>
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<tr>
<td>BLK</td>
<td>B-lymphoid kinase</td>
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<tr>
<td>BMX</td>
<td>Bone marrow-expressed kinase</td>
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<tr>
<td>BTK</td>
<td>Bruton’s Tyrosine Kinase</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C chemokine receptor</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
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<tr>
<td>CFSE</td>
<td>Carboxyfluorescein succinimidyl ester</td>
</tr>
<tr>
<td>CL</td>
<td>Cutaneous leishmaniasis</td>
</tr>
<tr>
<td>CLL</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
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<tr>
<td>CXCL</td>
<td>C-X-C chemokine ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>C-X-C chemokine receptor</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DLBCL</td>
<td>Diffuse Large B cell Lymphoma</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxy ribonucleic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HER</td>
<td>Human epidermal growth factor receptor</td>
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<tr>
<td>IC</td>
<td>Inhibitory concentration</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IRF</td>
<td>Interferon regulatory factor</td>
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<tr>
<td>ITK</td>
<td>IL-2 inducible T cell kinase</td>
</tr>
<tr>
<td>JAK</td>
<td>Jannus kinase</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MCL</td>
<td>Mantle Cell Lymphoma</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MM</td>
<td>Multiple myeloma</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PH</td>
<td>Pleckstrin homology</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PIP3</td>
<td>Phosphatidylinositol-3,4,5-triphosphate</td>
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<tr>
<td>PNPP</td>
<td>p-nitrophenyl phosphate</td>
</tr>
<tr>
<td>PRR</td>
<td>Proline-rich region</td>
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<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
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<tr>
<td>RLK</td>
<td>Resting lymphocyte kinase</td>
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<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real time-PCR</td>
</tr>
<tr>
<td>SH2</td>
<td>SRC homology domain 2</td>
</tr>
<tr>
<td>SH3</td>
<td>SRC homology domain 3</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
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<tr>
<td>Th cell</td>
<td>T helper cell</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>VL</td>
<td>Visceral leishmaniasis</td>
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<tr>
<td>WM</td>
<td>Waldenström’s macroglobulinemia</td>
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<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
<tr>
<td>XID</td>
<td>X-linked immunodeficiency</td>
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<tr>
<td>XLA</td>
<td>X-linked agammaglobulinemia</td>
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</table>
CHAPTER 1: INTRODUCTION

The immune system is our biological defense mechanism, with organs, cells and molecules, which work in a coordinated manner to generate an immune response and protect us against diseases. These protective mechanisms of the immune system were recognized in 1796 by Edward Jenner when he used a cowpox vaccination program to protect people from smallpox. 200 years since Jenner’s observations, we have witnessed a revolution in the field of medical intervention. The development of preventative and therapeutic vaccines and later, drugs such as antibiotics have allowed us to combat acute infectious diseases like rabies, measles, mumps, chickenpox etc. However, such dramatic successes have been limited for chronic diseases such as cancer and leishmaniasis where the cancer cell or parasite employs complex strategies to evade protective immune responses. Further advancements in our understanding of the molecular mechanisms of these diseases and innovations in targeted strategies that harness the power of the immune system, called ‘immunotherapies’, are critical for tackling such diseases.

My thesis focuses on one such immunotherapeutic strategy where we employ inhibitors against a protein called ‘Bruton’s tyrosine kinase’ to treat these diseases. Chapter 1 provides a brief historical perspective of targeted therapies, details on the function of Bruton’s tyrosine kinase in immune cells, how molecular inhibitors against this protein are currently employed for the treatment of cancer and two novel applications of these inhibitors which are the basis of my thesis. Since my thesis brings together
concepts from diverse fields, I have attempted in this chapter to provide a wide overview of the various topics needed to understand and interpret my scientific findings.

A historical perspective of targeted therapies – role of protein kinases

During past two decades, non-specific approaches for cancer treatment such as chemotherapy have given way to targeted therapies. The classical mode of cancer therapy, chemotherapy, focuses on disrupting essential cellular mechanisms. Many of the standard drugs used in chemotherapy interfere with DNA replication, DNA repair mechanisms and cell division in both cancer and healthy cells, leading to severe side-effects. On the other hand, targeted therapies, that are engineered to act on molecules specific to cancer cells, are attractive alternatives due to their specific mode of action. In the late twentieth century, two branches of targeted therapy emerged i.e. antibody therapy and single molecule inhibitors and they continue to dominate the current agenda for anti-cancer research.

In both branches of targeted therapies, the focus of research has been the signaling pathways that regulate the survival and proliferation of cancer cells. Figure 1.1 highlights the nodes in these pathways that have been intensely pursued in the past four decades. Many of these molecules belong to the kinase family of proteins – kinases are defined as enzymes that catalyze the transfer of phosphate molecules from high energy, phosphate donor molecules to substrates – which are often deregulated during cancers (Zhang, Yang, & Gray, 2009). In 1978, an intracellular kinase signaling protein from the avian sarcoma virus was discovered to play such a pro-oncogenic role in chickens (Collett & Erikson, 1978). A few years later in 1982, the activation of protein kinase C was found to be the mechanism of action of tumor-promoting phorbol esters (Castagna et al., 1982).
Additionally, the mutation or overexpression of kinase genes is frequently associated with many cancers in humans (Zhang et al., 2009). These studies demonstrate the critical role for these signaling molecules in the progression of cancer and as targets for treatment strategies. However, due to the low specificity and potency of available kinase inhibitors in the 1980s, there were limited advancements in their development for cancer therapy. In the meantime, antibodies that blocked kinase signaling pathways gained prominence for their potent anti-cancer activity. A receptor tyrosine kinase called human epidermal growth factor receptor (HER2) was found to be overexpressed in many patients with breast cancer. This discovery led to the development of monoclonal antibody Herceptin to target this protein for the treatment of HER2-positive metastatic breast cancers. Its subsequent FDA approval in 1998 revolutionized the field of targeted therapy (Huang, Shen, Ding, & Geng, 2014; Zhang et al., 2009). In 2001, the first small molecule inhibitor targeting a protein kinase for cancer treatment, imatinib, was approved. Imatinib targets Abelson tyrosine kinase (Abl) which is aberrantly expressed in patients with chronic myelogenous leukemia due to a chromosomal rearrangement event (Cohen, 2002). As of 2014, over 30 targeted drug therapies have been approved for the treatment of cancer, either alone or in combination with other drugs (Huang et al., 2014).

**Bruton’s tyrosine kinase - an important mediator of immune cell function**

Bruton’s tyrosine kinase (BTK) is a signaling molecule and a member of the TEC family of non-receptor tyrosine kinases (Figure 1.1). The TEC kinase family consists of 5 non-receptor tyrosine kinases expressed in various immune cells including TEC, BTK, ITK (IL-2 inducible T cell kinase), RLK (resting lymphocyte kinase) and BMX (bone marrow-expressed kinase). The structure of BTK, as is typical of the proteins in this
family, includes an amino-terminal pleckstrin-homology (PH) domain that recruits the protein to the plasma membrane, followed by a TEC homology domain containing proline-rich regions (PRRs), SRC homology (SH3) and SH2 domains and a carboxy-terminal kinase domain (Figure 1.2). Like other members of the TEC family, BTK functions in a two-step process. The first step involves activation of BTK in a complex cascade of molecular events. In response to upstream signaling events and phosphatidylinositol 3-kinase (PI3K) activation, BTK is sequestered to the membrane via its PH domain by binding to phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P₃). When BTK is recruited to the membrane, it undergoes inter- and intra-molecular conformational changes and is further activated by the phosphorylation activity of Src- or Syk-family kinases (Mohamed et al., 2009). After complete activation, the second step ensues where BTK catalyzes the phosphorylation of downstream signaling molecules. Depending on the immune cell where BTK is expressed and the signal transduction pathway that BTK is involved in, downstream signaling events have diverse physiological effects. More details regarding BTK’s role in the signaling pathways of different immune cells will be highlighted later.

In the past decade, several excellent reviews were published detailing the research on the role of the TEC family kinases in various immune cells (Berg, Finkelstein, Lucas, & Schwartzberg, 2005; Schmidt, Boucheron, Unger, & Ellmeier, 2004; Schwartzberg, Finkelstein, & Readinger, 2005). Immune cells can be categorized into two lineages: lymphoid and myeloid based on the progenitor cell from which they originate during hematopoiesis. Although BTK, like other members of the TEC family kinases, is differentially expressed in various hematopoietic cells (Schmidt et al., 2004), my thesis
will focus on the role of BTK in B cells, which are part of the lymphoid lineage, as well as myeloid cells.

**BTK plays a critical role in B cell development and function**

BTK is required for the development, homeostasis and function of B cells. Its importance in B cell development was first highlighted when mutations in the BTK gene were associated with a severe immunodeficiency disease in humans called X-linked agammaglobulinemia (XLA). Patients with this disease show low levels of circulating B cells and immunoglobulins and therefore, display limited humoral immunity required to ward off bacterial infections. Further research into the molecular mechanism indicated that BTK participates in B cell development pathway particularly during the early stages by playing a critical role in the signaling cascade of the pre-B cell receptor (pre-BCR). At this stage of B cell development, loss of BTK activity disrupts the survival signals provided by the assembled pre-BCR for further development of immature B cells leading to cell death and the diminished B cell counts in XLA patients. Apart from B cell development, recent research also demonstrates that basal functioning BCR signaling is required for relaying survival signals towards the homeostatic maintenance of mature resting B cells. When mature B cells encounter antigen during infection, BTK participation in BCR signaling promotes the survival and proliferation of B cells for the development of antibody response against the infection. Interestingly although BTK is expressed in various stages of B cell development and differentiation, it is not present in terminally differentiated plasma cells (Buggy & Elias, 2012; Mohamed et al., 2009; Seda & Mraz, 2015). BTK also participates in the signaling pathway for chemokine receptor, CXCR4, which is important for B cell migration. When CXCR4 binds to its cognate
ligand, CXCL12, it leads to activation of BTK and the downstream regulation of various adhesion molecules called integrins involved in homing of B cells (de Gorter et al., 2007).

**Targeting BTK for treatment of B cell malignancies**

Although the BCR pathway is crucial for the normal development and function of B cells, this pathway is also exploited by malignant cells of B cell origin to mediate pathogenesis. Many elegant studies over the years have systematically demonstrated how various B cell malignances such as chronic lymphocytic leukemia (CLL) show molecular signatures characteristic of activated BCR pathway, indicating their dependence on this pathway for survival signals (Bojarczuk et al., 2015; Zhong, Byrd, & Dubovsky, 2014). Since BTK is required for BCR signaling and loss of BTK activity diminishes BCR-activated signal transduction, targeted therapies have focused on this molecule for the treatment of B cell malignances (Aalipour & Advani, 2013).

To date, many single molecule inhibitors developed to target BTK have shown success in both pre-clinical and clinical studies. A highlight of the most promising inhibitors with a summary of their activity in various B cell malignancies has been presented in several recent reviews (Aalipour & Advani, 2013; Akinleye, Chen, Mukhi, Song, & Liu, 2013; Burger & Buggy, 2013; Zhong et al., 2014). Among the many BTK inhibitors, ibrutinib (marketed as Imbruvica) has shown tremendous results as a targeted therapy for certain types of B cell malignancies.
Ibrutinib, a Breakthrough Therapy Drug, for treatment of B cell malignancies

Ibrutinib is an irreversible inhibitor with a 50% inhibitory concentration (IC$_{50}$) of 0.5 nM for BTK (Burger & Buggy, 2013) and like most BTK inhibitors is an ATP-competitive inhibitor. BTK inhibitors mostly fall into two broad categories depending on their mechanism of action: reversible and irreversible. The C-terminal kinase domain of BTK (Figure 1.2) catalyzes the transfer of a terminal phosphate from high energy phosphate donor molecules such as ATP to target substrates. Both reversible and irreversible BTK inhibitors inhibit BTK activity by binding to this catalytic core and outcompeting ATP binding to this site. Reversible inhibitors form hydrogen bonds with residues in the core and thereby, inhibit the function of the enzyme (Zhang et al., 2009). On the other hand, irreversible inhibitors like ibrutinib inhibit BTK in a two-step process: the first step involves recognition and non-covalent bond formation with the core and in the second step, the inhibitor forms a covalent bond with a cysteine residue in the core. Since the ATP binding site is highly conserved among the TEC kinase family and the overall kinase family in general, most BTK inhibitors show off-target binding activity. However, ibrutinib is more selective compared to reversible inhibitors since its off-target activity mostly affects a much smaller subgroup of kinases in the human kinome that possess the cysteine moiety within the catalytic site including 4 TEC family kinases (ITK, TEC, BMX and RLK), 3 EGFR family kinases and kinases BLK and JAK3 (Q. Liu et al., 2013). Ibrutinib shows varying ability to bind irreversibly to these kinases, with highest affinity for BLK and BMX. Additionally ibrutinib binds to a few other kinases which do not possess a cysteine in their ATP-binding site in a reversible manner and such interactions are expected to be short-lived in vivo given short half-life of ibrutinib
following oral-dosing in humans (Burger & Buggy, 2013; Honigberg et al., 2010). Since ibrutinib was shown to potently inhibit the phosphorylation of BTK and downstream signaling targets in BCR stimulated B cells during \textit{in vitro} assays, it was tested for its ability to treat B cell malignancies (Burger & Buggy, 2013).

Earliest studies on the efficacy of ibrutinib in treating B cell malignancies showed that ibrutinib inhibited the survival of CLL cells from patients and delayed the progression of disease in a mouse model of CLL. Additionally, ibrutinib prevented actin polymerization and migration of CLL cells in response to tissue-homing chemokines CXCL12 and CXCL13 (Ponader et al., 2012). In a subtype of Diffuse Large B cell Lymphoma (DLBCL), another B cell malignancy, ibrutinib synergizes with an active agent against refractory/relapsed DLBCL called lenalidomide to inhibit chronic B cell signaling and thereby, kill DLBCL cells (Yang et al., 2012). In multiple myeloma (MM), a malignancy of plasma cells in the bone marrow, ibrutinib prevented the survival and migration of MM cells while also suppressing the secretion of MM cell-related growth factors, cytokines and chemokines (Tai et al., 2012). A clinical study on the effect of ibrutinib in patients with B cell malignancies demonstrated that patients with mantle cell lymphoma (MCL) and CLL treated with ibrutinib showed high response rates upon treatment with ibrutinib (Advani et al., 2013). Based on these studies, the U.S. Food and Drugs Administration (FDA) approved ibrutinib as a ‘Breakthrough Therapy Drug’ in November 2013 for the treatment of MCL patients who had received one prior therapy. It was additionally granted approval in 2014 for previously treated CLL patients as well as CLL patients with a specific mutation in chromosome 17. Another clinical trial demonstrating the efficacy of ibrutinib against a rare hematological malignancy called
Waldenström’s macroglobulinemia (WM) led to its recent expanded approval for the treatment of this disease as well (Treon et al., 2015). With ongoing research into the efficacy of ibrutinib against various forms of hematological malignancies, we can expect its continued expansion for supplemental indications.

**BTK is a crucial mediator of myeloid cell function**

Besides B cells, BTK is also expressed in various cells of the myeloid lineage such as neutrophils, macrophages and dendritic cells (Kawakami et al., 2006; Lachance, Levasseur, & Naccache, 2002; Melcher et al., 2008). In these cells BTK participates in integral early immune responses against diseases, called innate immune responses, such as Toll-like receptor (TLR) signaling, production of reactive oxygen and nitrogen species (ROS and RNS) and activation of T cell responses.

Myeloid cells use surface or intracellular receptors called Toll-like receptors (TLRs) to activate inflammatory responses upon detecting pathogens in the body. When TLRs bind to their respective ligands which are evolutionarily conserved molecular patterns on pathogens, this interaction leads to a cascade of signaling events which culminates in the nuclear localization of transcription factors and production of inflammatory cytokines to combat the infection (Kawai & Akira, 2009). Similar to its role in B cells where BTK transduces cell-surface signals downstream in the signaling cascade through its interaction with cell-membrane associated adaptor proteins and macromolecules, BTK interacts with various proteins proximate to the cell membrane during the TLR signaling pathway. BTK interacts with domains on TLRs 4, 6, 8 and 9 as well as with various adaptor proteins in the TLR4 signaling cascade. Studies using
myeloid cells from BTK deficient animal models and XLA patients show that it is critical for TLR-mediated cytokine production and that absence of BTK activity leads to a significant reduction in the secretion of inflammatory cytokines such as IL-12, TNF-α and IL-6 (Brunner, Müller, & Wirth, 2005; Mohamed et al., 2009; Schmidt et al., 2004). BTK deficiency also leads to poor oxidative burst as evidenced by a decrease in the production of nitric oxide and reactive oxygen intermediates by BTK deficient macrophages and neutrophils (Mangla et al., 2004). In dendritic cells (DCs), BTK appears to play an inhibitory role in the development and maturation of these cells. BTK deficient DCs express higher levels of MHC-II and co-stimulatory molecules upon TLR-4 stimulation and enhance activation of CD4+ T cells (Kawakami et al., 2006). Given the extensive research on how BTK regulates myeloid cell function, future studies should focus on how BTK’s involvement in myeloid cell effector function impacts the pathogenesis of diseases.

**Studying the effects of ibrutinib on myeloid cells could spur different applications**

In addition to its efficacy against B cell malignancies, ibrutinib is also effective in treating autoimmune diseases where pathogenesis is mediated by B cells and myeloid cells like neutrophils, monocytes and macrophages. During the early stages of ibrutinib development, scientists from Celera and Pharmacyclics Inc. reported that ibrutinib was primarily developed as a drug candidate for rheumatoid arthritis (Burger & Buggy, 2013). Research into its mechanism of action showed that ibrutinib dampens inflammatory responses during collagen antibody-induced arthritis by preventing the infiltration of neutrophils, monocytes, macrophages and lymphocytes into the site of inflammation. In this study, Chang et al. also showed that ibrutinib treatment on monocytes and
macrophages inhibits the secretion of cytokines like TNF-α and IL-6. Further, ibrutinib is effective against immune-complex diseases models where myeloid cells play a significant role in mediating inflammation (Chang et al., 2011). Ibrutinib also has the potential to be a drug candidate by targeting BTK in other disease models. In the case of acute myelogenous leukemia (AML), a cancer of myeloid cells, over 80% of patients show BTK expression in leukemic cells. Recent research has shown that ibrutinib inhibits BTK activity in AML cells and thereby, suppresses the proliferation and migration of these cells (SA Rushworth, Murray, Zaitseva, Bowles, & MacEwan, 2014; Stuart a Rushworth et al., 2015). Ongoing clinical trials will be informative about the efficacy of ibrutinib in treating AML. Since ibrutinib clearly modulates the function of myeloid cells, more research is required to better understand its effects on the immune responses of different types of myeloid cells.

**Thesis outline**

My focus of my thesis is two-fold. Chapter 2 describes how ibrutinib modulates the development and activation of dendritic cells, which are part of the myeloid lineage. To date, the effects of ibrutinib have not been sufficiently explored in this immune cell. Chapter 3 shows that ibrutinib is effective in suppressing the progression of a parasitic disease called leishmaniasis. These results are an exciting new indication for ibrutinib in the treatment of infectious diseases. Taken together, both these studies demonstrate novel effects of ibrutinib and have the potential to expand ibrutinib for new applications.
In the past decade, many promising targets have emerged for cancer therapy. The earliest discovered oncogenic proteins were growth factor receptors and signaling molecules and they continue to be heavily researched as targets for cancer treatments.

Recently, apoptotic proteins, epigenetic factors and angiogenesis regulators have also gained prominence for their potential in regulating cancer survival and metastasis. Advances in cancer immunology have led to the discovery of immunotherapeutic targets such as immune checkpoint proteins which can be regulated to activate the immune system to fight the cancer cells. (Adapted from Dr. Min Huang (Huang M. 2014))
Figure 1.2. Structure of TEC family of kinases. Typical structure of TEC kinases includes an N-terminal membrane binding domain (PH), followed by a proline rich TEC homology domain (TH), SRC homology domain 3 (SH3), SH2 domain and a C-terminal catalytic kinase domain. In ITK, TEC, BTK and BMX, the N-terminal pleckstrin-homology domain recruits these proteins to the membrane upon activation. RLK is anchored to the membrane constitutively by a N-terminal cysteine rich domain.
CHAPTER 2: TREATING BONE MARROW DERIVED DENDRITIC CELLS WITH A BTK INHIBITOR IBRUTINIB ENHANCES THEIR MATURATION, TLR-MEDIATED ACTIVATION AND ABILITY TO PROMOTE T HELPER RESPONSES.

Abstract

BTK is expressed in murine dendritic cells (DCs) and plays a role in the development of DCs. Further, BTK participates in TLR-4 signaling and modulates the ability of DCs to activate T cell responses. Hence, we evaluated whether ibrutinib treatment during the development of DCs from murine bone marrow precursors alters the development and activation status of these cells. Ibrutinib treatment promoted the development of CD11c+ DCs and enhanced the expression of DC activation markers such as MHC-II and co-stimulatory molecules like CD80. We also studied whether ibrutinib treatment modulates the response of DCs to stimulation with TLR-4 ligand, LPS. When ibrutinib-treated DCs were stimulated with LPS, they displayed further increase in DC activation markers and enhanced the production of cytokines like IFN-β and IL-10 compared to untreated DCs. Ibrutinib treatment also led to an increase in the expression of CCR7, a chemokine receptor which is critical for the migration of mature DCs to T cell specific areas in the lymph node. Further, LPS/ibrutinib-treated DCs promoted higher rates of CD4+ T cell proliferation and T cell derived cytokine production compared to
LPS-only stimulated DCs. Since ibrutinib has been previously shown to target another TEC kinase, ITK, we studied whether the enhanced maturation and LPS-mediated activation of DCs by ibrutinib was a result of inhibition of ITK, by using an ITK selective inhibitor, PRN694. Our results showed that enhanced maturation and activation of DCs is not mediated by the inhibition of ITK. We also observed that ibrutinib treatment in differentiated DCs altered cytokine responses following LPS stimulation and also enhanced DC-mediated T cell activation. These results indicate that ibrutinib plays a dual role in modulating both development as well as TLR-4 signaling in DCs. Finally, ibrutinib enhanced the expression of Type I interferons, like IFN-α and IFN-β, as well as transcription factors involved in signaling of Type I IFNs such as IRF-7 during the developmental process. Ibrutinib treatment also differentially regulated the expression of various cytokines and transcription factors involved in the development of DCs. In summary, our results show that ibrutinib enhances the maturation and activation of DCs, which provide a rationale for employing ibrutinib in the development of DC-based therapies. Further, our results also show that ibrutinib alters the expression of genes involved in the development of DCs. Future studies should focus on uncovering the mechanisms underlying this process.

Introduction

Dendritic cells (DCs), a subset of the myeloid lineage, are a part of the innate immune system. They act as sentinels of the immune system by surveying their environment for potential antigenic stimuli. Additionally, DCs have superior antigen processing abilities; they present antigen to naïve T cells using MHC-I and MHC-II molecules and initiate T cell responses by the production of cytokines and the expression
of co-stimulatory molecules on their cell surface (Figure 2.1). This ability of DCs to activate T cells is critical for the development of an antigen-specific adaptive immune response to eliminate the target antigen (Gabrilovich, Ostrand-Rosenberg, & Bronte, 2012; Palucka & Banchereau, 2012; Wallet, Sen, & Tisch, 2005). The importance of DCs for the generation of a strong T cell response is evident in diseases like cancer where the recruitment of DCs is critical for resistance. In such diseases, DCs initiate the development of anti-cancer immune response by presenting tumor-derived antigens to T cells and activating tumor-specific T cell response (Diamond et al., 2011; Fuertes et al., 2011). Although tumor-derived factors can recruit DC precursors to the cancer microenvironment (Palucka & Banchereau, 2012), the presence of DCs in the tumor microenvironment is not necessarily associated with the generation of an anti-cancer immune response. As the disease progresses, cancer cells can create an immunosuppressive microenvironment by decreasing the presence of mature, functionally competent DCs and promoting the accumulation of immature DCs that facilitate tumor progression. Along with other immunosuppressive cells in the tumor microenvironment, immature DCs promote the differentiation of T cell populations such as regulatory T cells (Treg) and T helper 2 (Th2) cells which impede the development of tumoricidal immune responses rather than generate a potent anti-cancer T helper 1 (Th1) cell and cytotoxic T lymphocyte (CTL) response. Hence, drugs that promote the maturation of DCs and thereby, reprogram the tumor microenvironment to generate robust anti-cancer T cell responses are currently a focus of research in the field of cancer therapeutics (Gabrilovich et al., 2012; Palucka & Banchereau, 2012).
Besides targeting malignant B cells in hematological malignancies, ibrutinib has also been shown to act on other BTK-expressing immune cells such as myeloid cells. Research by Chang et al. shows that ibrutinib modulates the recruitment and cytokine responses of myeloid cells like monocytes, macrophages and neutrophils during immune complex models of disease (Chang et al., 2011). Hence, ibrutinib could modulate the function of other BTK-expressing myeloid cells like DCs.

A previous report by Kawakami et al indicates that BTK plays an inhibitory role during the development of DCs. Furthermore, BTK deficiency enhances the maturation of DCs by promoting the expression of surface markers upon LPS stimulation and leading to higher rates of CD4⁺ T cell activation (Kawakami et al., 2006). Since lack of BTK activity enhances the maturation of DCs, we wanted to study whether the inhibition of BTK using ibrutinib would have a similar effect on DC maturation. We evaluated this by culturing murine bone marrow cells in the presence of DC polarizing growth factors, with or without ibrutinib to generate ibrutinib-treated and untreated DCs respectively. We compared the activation status, LPS-induced immune responses and DC-mediated T cell activation by ibrutinib-treated and untreated DCs. Our studies have the potential to spark novel applications for ibrutinib in field of DC-based therapies for cancer treatment.

**Materials and Methods**

**Mice strains.** Female C57BL/6 mice (age 8-10 weeks) and OT.II TCR transgenic mice (age 8-10 weeks) were purchased from Harlan and Jackson Laboratories respectively. All animals were housed in a pathogen-free facility in The Ohio State University in accordance with National Institutes of Health and institutional guidelines.
Cultivation of bone marrow derived dendritic cells, drug treatments and TLR-activation studies. Bone marrow derived dendritic cells (DCs) were cultivated as described previously (Inaba et al., 1992; Natarajan et al., 2015). Briefly, bone marrow cells were isolated from femurs and tibias of mice, treated with ACK lysis buffer (0.15M NH₄Cl, 10mM KHCO₃, 0.1mM Na₂EDTA, pH 7.2 – 7.4) to lyse red blood cells, washed twice with sterile PBS (Gibco) and plated in 100 X 15 mm polystyrene, vented petri dishes @ 5 million cells per plate along with complete RPMI (RPMI medium (Gibco) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 1% penicillin (20 Units/ml)/streptomycin (20 µg/ml) (Life Technologies) and 1% HEPES) and 20 ng/ml GMCSF (granulocyte-macrophage colony stimulating factor) (Peprotech) for 7 days. At day 7, DCs were collected by gently harvesting the cells in the floating fraction of the culture medium to obtain greater than 60% purity of CD11c⁺ DCs in the floating fraction. The remaining cells in this fraction did not display lineage specific markers for T cells, B cells or NK cells upon analysis by flow cytometry. Approximately 30% of the total cell population was determined to be CD11b⁺ Ly6C⁺ monocytes upon flow cytometric analysis. A small percentage of CD11b⁺ Ly6G⁺ neutrophils were also observed (approximately 6-8% of total cell population) but were observed to die when cells were rested overnight. After harvesting DCs from the floating fraction, cells were washed once with sterile PBS prior and analyzed for the expression of surface molecules by flow cytometry. In order to evaluate phagocytic ability and the immune responses generated upon TLR stimulation, DCs were seeded @ 0.5 million cells per well in 24-well plates (Corning Life Sciences) and rested for 24 hours prior to further experiments.
**Drug treatments.** For experiments studying the effect of ibrutinib on DC development, cultures were treated with 1 μM ibrutinib (Pharmacyclics Inc.) or PBS at day 1 and remained in the supernatant until the end of the culture period to generate ibrutinib-treated and untreated DCs respectively. For experiments studying the effect of PRN694 on DC development, cultures were treated with 0.2 μM PRN694 (Principia Biopharma Inc.) or PBS at day 1 and remained in the supernatant until the end of the culture period to generate PRN694-treated and untreated DCs respectively. For experiments studying the effect of ibrutinib on differentiated DCs, rested DCs were washed once with sterile PBS and treated with DMSO (control) or 1 μM ibrutinib (Pharmacyclics Inc.) for 30 minutes at 37°C and 5% CO₂.

**Parasite uptake study.** Rested untreated and ibrutinib-treated DCs, generated by culturing developing DCs with GM-CSF without or with 1 μM ibrutinib (Pharmacyclics Inc.) as indicated above, were washed once with sterile PBS and infected with 2 million stationary phase, red fluorescent *Leishmania donovani* parasites as indicated previously (Dey et al., 2014). Parasite infection was performed for 3, 6 and 24 hours at 37°C and 5% CO₂. At the respective time points, cells were harvested, washed once with sterile PBS and stained with fluorescently labelled antibody for CD11c (Biolegend) prior to analysis of parasite uptake by flow cytometry (FACS Calibur, BD Biosciences). Data analysis was performed using Flowjo Software (Tree Star, Inc.) and percentage of infected DCs was determined by gating on CD11c⁺ Leishmania⁺ cells.
**LPS activation studies.** Control or drug treated DCs, generated as mentioned above, were washed twice with sterile PBS prior to conducting TLR-activation studies. For LPS stimulation studies, DCs were treated with control (media) or 1 µg/ml LPS (Sigma Aldrich) at 37°C and 5% CO₂ for 18, 24 or 48 hours. At 18 hours of LPS stimulation, RNA was extracted from cells to conduct real-time PCR analysis. At 24 hours of LPS stimulation, culture supernatants were harvested to measure cytokine production by ELISA and cells were isolated for analysis of surface activation markers by flow cytometry. At 48 hours of LPS stimulation, culture supernatants were harvested to measure nitric oxide production by Griess assay.

**DC in vivo migration study.** DC in vivo migration study was conducted as mentioned previously with some modifications (Martin-Fontecha et al., 2003). Briefly, untreated and ibrutinib-treated DCs, generated by culturing developing DCs with GM-CSF without or with 1 µM ibrutinib (Pharmacyclics Inc.) as indicated above, were seeded @ 3 million per well and rested for 24 hours. Rested DCs were washed once with sterile PBS and stimulated with 1 µg/ml LPS (Sigma Aldrich) at 37°C and 5% CO₂ for 24 hours. After 24 hours, DCs were harvested, washed once with sterile PBS, stained with 5 µM CFSE (Rotta et al., 2003) (Life Technologies) and subcutaneously injected into the footpads of C57BL/6 mice. After 72 hours, draining popliteal lymph nodes were harvested from mice and single cell suspensions were prepared. Lymph node cells were stained with fluorescently labelled antibody for CD11c (Biolegend) to analyze the numbers of migrated DCs by flow cytometry. Analyses were conducted by gating on CD11c⁺ CFSE⁺ DCs.
**T cell co-culture and proliferation studies.** Drug treated DCs were pulsed with 10 µg/ml OVA-peptide (323-339) (Anaspec) for 2 hours prior to treatment with 1 µg/ml LPS (Sigma Aldrich) for 22 hours at 37°C and 5% CO₂. After OVA/LPS stimulation, DCs were washed twice with sterile PBS and cultured with CFSE-stained T cells enriched from splenocytes of OT-II mice as described previously (Natarajan et al., 2015). Briefly, spleens from OT-II mice were mashed to prepare single cell suspensions, treated with ACK Lysis buffer to remove red blood cells and incubated in nylon wool columns for one hour at 37°C and 5% CO₂ to remove B cells by adherence to nylon wool (Hathcock, 2001). After incubation, T cell-enriched fraction was prepared by eluting cells from the column and contained >90% CD3⁺ T cells. B cell-enriched fraction was prepared by plunging column with media and contained >65% B220⁺ B cells and approximately 25% CD3⁺ T cells. For experiments where DCs were co-cultured with T cells, cells from T cell fraction were washed once with sterile PBS, stained with 5 µM CFSE (Life Technologies) and cultured with OVA, OVA/LPS, OVA/drug, OVA/LPS/drug-treated DCs in 1:4 ratio (DC:T cells) for 4 or 6 days. After 4 and 6 days of co-culture, proliferation of CD4⁺ T cells was determined by measuring the percentage of CD4⁺ CFSE<sub>low</sub> proliferating T cells by flow cytometry. Culture supernatants were harvested at day 4 and 6 of co-culture to determine the concentration of cytokine by ELISA. In experiments studying the effect of B cells in the DC-T cell co-culture, OVA pulsed LPS-untreated and LPS/ibrutinib-treated DCs were cultured in 1:4 ratio each with T cells, B cells or both for 6 days to measure cytokines in the culture supernatants by ELISA.
**Real-time PCR analysis.** Total RNA from developing DCs or LPS-stimulated DCs was extracted by adding TRIzol reagent to cells (Life Technologies) and performing chloroform-isopropanol extraction of RNA according to the manufacturer’s protocol. RNA was washed with 75% ethanol and resuspended in 1X TE buffer (Life Technologies). RNA concentration was determined by absorbance at 260nm. One µg RNA was used for first strand cDNA synthesis with SuperScript VILO cDNA synthesis kit (Invitrogen). Primer sequences and cycling conditions were obtained from PRIMER BANK (Wang, 2003). PCR amplification was conducted in an Opticon Real-Time PCR cycler (Biorad) using SYBR Green (BioRad) for detection. Data were normalized to β-actin and represented as fold induction over control only-treated cells by ∆∆CT method.

**Flow cytometry.** Cells were washed with once with PBS, blocked using normal mouse serum and incubated with conjugated antibodies against various cell surface markers including CD11c, Ly6C, MHC-II, CD40, CD80, CD86 and CCR7 (Biolegend). Cells from co-cultures assays were washed and blocked as mentioned above and incubated with conjugated antibody against CD4 (Biolegend). Samples were acquired on a BD FACS Calibur (BD Biosciences). Data analysis was performed using FlowJo software (Tree Star, Inc.). During analysis, gating was performed based on respective isotype controls for the corresponding conjugated antibody. Analysis of surface marker expression on DCs was performed by gating on CD11c+ cells. Percentage of proliferating cells in T cell co-culture assay was measured by gating on CD4+ T cells and measuring the percentage of CFSElow cells using the FlowJo software.
**Cytokine ELISA.** Cytokine ELISA was performed on culture supernatants of LPS-stimulated DCs and co-cultures as described previously (Oghumu et al., 2014). Briefly, purified anti-mouse IL-10, IL-6, TNF-α, IL-12, IFN-γ, IL-17 (Biolegend), IFN-β and IL-13 (eBiosciences) monoclonal antibodies were used as capture antibodies for the respective ELISA. Recombinant mouse IL-10, IL-6, TNF-α, IL-12, IFN-γ, IL-13 (BD Biosciences), IFN-β and IL-17 (eBiosciences) were used as standards. Detection of cytokines was performed using biotinylated anti-mouse antibodies for IL-10, IL-6, TNF-α, IL-12, IFN-γ, IL-17 (Biolegend), IFN-β and IL-13 (eBiosciences), streptavidin conjugated alkaline phosphatase (BD Pharmingen) and p-nitrophenyl phosphate (PNPP) tablets (Thermo Fisher Scientific) as substrate. Plates were read using Spectramax M3 microplate reader (Molecular Devices LLC) at an absorbance of 405 nm. Cytokine concentrations were determined by extrapolation from the generated standard curve using Softmax Pro software (Molecular Devices LLC).

**Griess assay.** Culture supernatants of LPS-stimulated DCs were tested for the presence of nitric oxide as described previously (Sanchez et al., 2010). Briefly, nitrite was measured in culture supernatants using Griess Reagent (Sigma Aldrich) with sodium nitrite as the standard. Plates were read using a Spectramax M3 microplate reader (Molecular Devices LLC) at the absorbance of 570 nm. The concentrations of nitric oxide were determined by extrapolation of generated standard curves using Softmax Pro software (Molecular Devices LLC).
Statistical analysis. All statistical analyses were done using Prism 5 (GraphPad Software). Student’s unpaired t test was employed to determine statistical significance of values obtained. The p values less than 0.05 were considered statistically significant.

Results

Ibrutinib treatment enhances the development and maturation of DCs.

In order to study whether inhibition of BTK using ibrutinib treatment affects the development of DCs, we cultured mouse bone marrow cells with a growth factor GM-CSF to promote the differentiation of DCs and treated the cells with ibrutinib or PBS during the developmental process to generate ibrutinib-treated and untreated DCs respectively. We compared the proportion of DCs in ibrutinib-treated and untreated DC cultures by measuring the expression of CD11c, a surface integrin used as a marker for DCs, by flow cytometry. We observed that ibrutinib-treated DC cultures had a significantly higher percentage of CD11c+ DCs compared to untreated DC cultures (Figure 2.2A).

In order to compare the maturation status of the DCs generated in ibrutinib-treated and untreated DC cultures, we measured the expression of surface molecules that are differentially regulated during the maturation of DCs. When DCs undergo maturation, they downregulate the expression of Ly6C and enhance the expression of MHC-II and co-stimulatory molecules CD80 and CD86 (Caton, Smith-Raska, & Reizis, 2007; Lutz & Schuler, 2002). We observed that ibrutinib treatment increased the percentage of MHC-II+ and CD80+ DCs and decreased the percentage of Ly6C+ and CD86+ DCs (Figure 2.2B). Further, ibrutinib-treated DCs displayed higher cell-surface expression levels of
MHC-II and CD80 and lower expression levels of Ly6C and CD86 compared to untreated DCs (Figure 2.2C). As DCs become mature, they lose their ability to perform phagocytosis efficiently (Mellman & Steinman, 2001). We evaluated the phagocytic capacity of ibrutinib-treated and untreated DCs by measuring their ability to engulf red fluorescent *Leishmania* parasites using flow cytometry. At 3, 6 and 24 hrs post-infection, ibrutinib-treated DCs had significantly lower proportion of infected cells compared to untreated DCs, although both ibrutinib-treated and untreated DCs showed a steady increase in uptake of parasites at higher incubation times (Figure 2.3). Taken together our results indicate that inhibition of BTK using ibrutinib enhances the development and maturation of bone marrow derived DCs.

**Ibrutinib treatment enhances the expression of MHC-II and CD80 while reducing the expression of CD86 upon LPS stimulation in DCs.**

DCs sense pathogens in the body by using intracellular or extracellular pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) which recognize evolutionarily conserved molecular patterns in pathogens. Ligands to TLRs are also being evaluated as adjuvants for DC-based cancer therapies (Palucka & Banchereau, 2012). An example of a PRR is TLR-4 which recognizes lipopolysaccharide (LPS), an abundant sugar on the surface of bacterial cell walls. When LPS binds to TLR-4, this interaction stimulates DCs to produce cytokines, upregulate the expression of MHC-II and co-stimulatory molecules and activate T cell responses (Diebold, 2009). We studied whether ibrutinib-treated DCs would respond more robustly to LPS-mediated activation by comparing the expression of MHC-II and co-stimulatory molecules such as CD80, CD86
and CD40 in ibrutinib-treated and untreated DCs. We observed that ibrutinib treatment increased the percentage of MHC-II$^+$ and CD80$^+$ DCs and decreased the percentage of CD86$^+$ DCs upon LPS stimulation (Figure 2.4A). Additionally, LPS/ibrutinib-treated DCs upregulated the expression levels of MHC-II and CD80 and downregulated expression levels of CD86 compared to LPS/untreated DCs (Figure 2.4B). We did not find any significant difference in the percentage of CD40$^+$ DCs or levels of CD40 between LPS/ibrutinib-treated and LPS/untreated DCs (Figure 2.4A – 2.4B).

Ibrutinib regulates the expression of chemokine receptors and promotes the migration of LPS-activated DCs.

In addition to upregulating surface activation molecules and producing cytokines, DCs differentially regulate the expression of various chemokine receptors during the process of maturation and activation. Immature DCs show higher expression of chemokine receptors such as CCR2, CCR5, CCR6 and CXCR2 which mediate trafficking of DCs to sites of inflammation (McColl, 2002). However, upon LPS-mediated activation and maturation, DCs upregulate the expression of a chemokine receptor, CCR7, to migrate from peripheral sites of the body into T cell specific regions within the draining lymph node and induce T cell activation (Randolph, 2001). In order to better understand how ibrutinib regulates the expression of these chemokine receptors, we compared the induction of these genes during the development of ibrutinib-treated and untreated DCs using RT-PCR. We did not observe any significant differences in CCR2 and CCR5 expression between untreated and ibrutinib-treated DC cultures although there was a trend towards higher expression of these genes in ibrutinib-treated DC cultures at the
early time point (Figure 2.5A and 2.5B). Similarly, there was a trend towards higher expression of CCR6 in ibrutinib-treated DC cultures even though this gene was significantly downregulated at the early time point (Figure 2.5C). On the other hand, CXCR2 was significantly dampened in ibrutinib-treated DC cultures at the later time point (Figure 2.5D).

We also evaluated whether LPS stimulation would enhance the surface expression of CCR7 in ibrutinib-treated DCs. We observed a higher percentage of CCR7+ DCs in LPS/ibrutinib-treated DC cultures compared to LPS/untreated DC cultures (Figure 2.6A). We also compared the migration of ibrutinib-treated and untreated DCs upon LPS stimulation. We labelled LPS/ibrutinib-treated and LPS/untreated DCs with a fluorescent dye called CFSE to track the cells and injected them subcutaneously in the footpads of mice. After 72 hours, we harvested draining lymph nodes and measured the proportion of CD11c+ CFSE+ DCs in mice injected with LPS/ibrutinib-treated and LPS/untreated DCs. We observed that mice injected with LPS/ibrutinib-treated DCs had higher numbers of CFSE-labelled DCs in the draining lymph nodes compared to mice injected with LPS/untreated DCs (Figure 2.6B). Taken together, these results indicate that ibrutinib regulates the expression of various chemokine receptors. Further, ibrutinib promotes the expression of CCR7 and enhances the migration of LPS-activated DCs in vivo.

**Ibrutinib differentially regulates the production of cytokines upon stimulation with LPS.**

In order to further characterize how ibrutinib-treated DCs respond to LPS-mediated activation, we compared LPS-induced cytokine and nitric oxide responses in
ibrutinib-treated and untreated DCs. We observed that LPS/ibrutinib-treated DCs produced higher levels of IL-10 and IFN-β (Figure 2.7A and 2.7B) and lower levels of IL-6, IL-12 and nitric oxide (Figure 2.7C – 2.7E). There was no significant difference in TNF-α production between LPS/ibrutinib-treated and LPS/untreated DCs (Figure 2.7F).

**Treatment with ibrutinib enhances the ability of DCs to activate CD4+ T cell responses.**

Upon detecting pathogens, DCs engage the adaptive immune system in order to effectively combat the infection. CD4+ T cells are a critical arm of the adaptive immune response during infection since they produce cytokines to recruit and activate various immune cells to clear the pathogens. CD4+ T cells undergo activation by three key signals from DCs (Figure 2.1). Firstly, T cells recognize phagocytosed antigen presented by DCs via MHC-II molecules to their T cell receptor. Secondly, T cells are activated to proliferate by the ligation of co-stimulatory molecules on surface of DCs with corresponding receptors on the T cell surface. And thirdly, the milieu of cytokines produced by DCs and other immune cells in the microenvironment of the T cell, along with the ligation of appropriate co-stimulatory molecules direct the differentiation of naïve T cells into specific T helper cell subsets depending on the type of infection (Diebold, 2009). Since ibrutinib differentially regulates the expression of MHC-II, co-stimulatory molecules and cytokine production upon LPS stimulation (Figure 2.4 and 2.7), we studied whether this would impact the ability of ibrutinib-treated DCs to activate CD4+ T cell responses. We studied this using an ovalbumin (OVA) peptide antigen specific DC: T cell co-culture model. We observed that LPS/ibrutinib-treated DCs
promoted higher rates of T cell proliferation compared to LPS/untreated DCs (Figure 2.8A). We also measured the levels of cytokines in the co-culture supernatants. While LPS/ibrutinib-treated DCs enhanced IFN-γ and IL-13 production at earlier and later time points compared to LPS/untreated DCs, IL-17 production was significantly higher at a later time point (Figure 2.8B – 2.8D). We also evaluated whether the presence of B cells in the co-culture altered the ability of LPS/ibrutinib-treated DCs to enhance T cell responses. We observed that LPS/ibrutinib-treated DCs promoted T cell proliferation compared to LPS/untreated DCs even in the presence of B cells (data not shown). However, the presence of B cells led to significant reduction in the production of IFN-γ in the LPS/ibrutinib-treated DC co-culture while there were no differences in the levels of IL-13 and IL-17 between LPS/ibrutinib-treated and LPS/untreated DC co-cultures (Figure 2.8E – 2.8G). Taken together, our results indicate that treatment with ibrutinib enhances the ability of DCs to activate CD4⁺ T cell responses. Further, the presence of B cells in the DC-T cell co-culture modulates cytokine profile induced by ibrutinib treatment.

Enhanced maturation and LPS-mediated activation of DCs upon ibrutinib treatment is not dependent on inhibition of ITK.

Ibrutinib binds to and inhibits the activity of another Tec kinase ITK in addition to its activity on BTK (Dubovsky et al., 2013). Although the effects of ibrutinib on enhancing the maturation and activation of DCs can be explained by its inhibition of BTK, we wanted to evaluate whether inhibition of ITK could explain the enhanced maturation and activation of DCs observed in our studies. It is not known whether ITK is
expressed by DCs during the process of development or by mature DCs. Hence we evaluated the expression of ITK in developing DCs as well as mature DCs. Although cells in the developing DC culture expressed ITK, it was not expressed by mature DCs (data not shown). It possible that ITK is expressed by DC precursors and therefore, plays a role in the development of DCs. Using PRN694, an ITK selective inhibitor, we evaluated whether inhibiting the function of ITK during the development of DCs contributes to enhanced maturation and LPS-mediated activation. We treated bone marrow cells with GM-CSF and cultured the cells with PBS or PRN694 during the developmental process to generate PRN694-treated and untreated DCs respectively. When we compared the proportion of CD11c+ DCs in the two cultures, we observed that there was significantly lower percentage of CD11c+ DCs in PRN694-treated DC culture compared to untreated DC culture (Figure 2.9A). Further, there was significantly lower percentage of MHC-II+ and CD80+ DCs and higher percentage of Ly6C+ DCs in PRN696-treated DC culture compared to untreated DC culture (Figure 2.9B). PRN694-treated DCs also showed reduced expression levels of MHC-II and CD80 and increased expression levels of Ly6C (Figure 2.9C). When we studied the production of cytokines upon LPS stimulation by PRN694-treated and untreated DCs, we observed that there was significantly higher IL-6 production by LPS/PRN694-treated DCs compared to LPS-only treated DCs, but IL-10, IL-12 and TNF-α synthesis was similar between the two groups (Figure 2.10A - 2.10D). We also measured the ability of LPS/PRN694-treated DCs to activate CD4+ T cell responses compared to LPS-only treated DCs. We observed that there were no differences in the ability of LPS/PRN694-treated DCs and LPS-only treated DCs to promote T cell proliferation (Figure 2.10E) and T cell derived cytokine
responses (data not shown). Hence, taken together our results show that enhanced maturation and activation of DCs is not mediated by the inhibition of ITK.

**Ibrutinib modulates TLR-4 signaling and DC-mediated T cell activation in differentiated DCs.**

Previous studies show that BTK participates in TLR-4 signaling in myeloid cells such as DCs, in addition to its role in the development of DCs (Brunner et al., 2005; Kawakami et al., 2006). In order to better understand ibrutinib’s dual role in the development and TLR-4 signaling in DCs, we decided to investigate TLR-4 responses in differentiated DCs upon ibrutinib treatment. In order to study this, we generated DCs by culturing mouse bone marrow cells in the presence of GM-CSF and treated the differentiated DCs with PBS/ibrutinib to generate ibrutinib-treated and untreated DCs. We examined whether ibrutinib modulates cytokine and nitric oxide (NO) production in differentiated DCs upon LPS stimulation. Ibrutinib-treated DCs displayed dampened TNF-α and NO production compared to untreated DCs upon LPS stimulation (Figure 2.11A and 2.11C), while IL-12 production was comparable between both groups (Figure 2.11B). Further, IL-6, IL-18 and TGF-β expression was significantly enhanced upon LPS/ibrutinib treatment compared to LPS-only treatment (Figure 2.11D -2.11F). There was a trend towards higher IL-1β expression in LPS/ibrutinib-treated DCs compared to LPS/control-treated DCs but this difference was not statistically significant (data not shown).

We then determined how ibrutinib affects the expression of MHC-II and co-stimulatory molecules upon LPS stimulation in differentiated DCs. Ibrutinib treatment
reduced the percentage of MHC-II\(^{+}\) and CD86\(^{+}\) cells and increased the percentage of CD80\(^{+}\) cells compared to PBS treatment in LPS-stimulated DCs (Figure 2.12A). Further, LPS/ibrutinib-treated DCs displayed lower levels of MHC-II and CD86 expression and higher CD80 expression compared to LPS/control-treated DCs (Figure 2.12B). However, percentages of CD40\(^{+}\) DCs and expression levels of CD40 remained similar between LPS/ibrutinib- and LPS/control-treated DCs (data not shown).

DC-derived cytokines such as IL-6 and TGF-β are critical in initiating IL-17 production by T cells (Walsh & Mills, 2013). Based on the cytokine profile generated upon ibrutinib treatment (Figure 2.11), we hypothesized that ibrutinib-treated DCs would promote an IL-17 response from T cells. We investigated this by using an in vitro antigen-specific DC:T cell co-culture model. T cells co-cultured with LPS/ibrutinib-treated DCs displayed higher proliferation rates compared to T cells co-cultured with LPS/control-treated DCs (Figure 2.13A). We also evaluated the production of T cell cytokines in the co-culture supernatants. LPS/ibrutinib-treated DCs enhanced the production of IL-17, but not IFN-γ or IL-13 compared to LPS/control-treated DCs (Figure 2.13B – 2.13D). We could not detect the presence of IL-4 in either treatment (data not shown).

In summary, our results indicate that ibrutinib treatment on differentiated DCs modulates TLR-4 signaling to promote an IL-17 response by CD4\(^{+}\) T cells.
Ibrutinib modulates the expression of transcription factors and cytokines that regulate the development of dendritic cells.

The maturation of DCs is regulated by various cytokine and transcription factors that are produced and/or activated during the developmental process (K. Liu & Nussenzweig, 2010; Merad, Sathe, Helft, Miller, & Mortha, 2013; Mohty et al., 2003; Moore & Anderson, 2013). Since our studies indicate that DCs treated with ibrutinib during the course of development are more mature than untreated DCs, we evaluated whether ibrutinib modulates the expression of these cytokines and transcription factors involved in DC development. We studied this by comparing the levels of mRNA for the respective genes between ibrutinib-treated and untreated DCs at days 4 and 7 of culture using RT-PCR. Type I Interferons, such as IFN-α and IFN-β produced by bone marrow derived DCs, are critical for their phenotypic and functional maturation (Montoya et al., 2002). Hence, we first evaluated whether ibrutinib treatment leads to increased expression of IFN-α and IFN-β during the development of DCs. Ibrutinib treatment induced a three-fold increase in the expressions of IFN-α (Figure 2.14A) and IFN-β (Figure 2.14B) at day 7 of culture. Since interferon regulatory factors (IRFs) mediate the signaling as well as the production of Type I IFNs, we evaluated the expression of various IFNs known to mediate signaling of Type I IFNs in myeloid dendritic cells (Lazear et al., 2013; Moore & Anderson, 2013). We observed a 50% increase in the expressions of IRF-1 and IRF-5 at day 4 in ibrutinib-treated DCs. However, there were no differences in the expressions of these two transcription factors at day 7 of culture between ibrutinib-treated and untreated DCs (Figure 2.14C and 2.14E). Further, the expression of IRF-3 was similar between ibrutinib-treated and untreated DCs at both day
4 and 7 of culture (Figure 2.14D). Interestingly, there was a two-fold increase in the induction of IRF-7 upon ibrutinib treatment compared to no treatment at day 7 which highly correlated with the increase in IFN-α and IFN-β expressions at the same time point (Figure 2.14F). These results suggest that the enhanced DC maturation upon ibrutinib treatment could be mediated by the upregulation of IFN-α, IFN-β and IRF-7 during their developmental process.

Various STAT (Signal transducer and activator of transcription) proteins are required for the development of myeloid cells (Smithgall et al., 2000). Hence, we evaluated whether ibrutinib regulates the expression of STATs during the development of DCs. We observed that there was significantly higher induction of STAT2 (1.75-fold, Figure 2.15B), STAT5A (2-fold, Figure 2.15D) and STAT6 (1.5-fold, Figure 2.15F) upon ibrutinib treatment at day 4 of culture, but no difference in mRNA levels of these STATs at day 7 of culture. There was a trend towards higher expression of STAT1 in ibrutinib-treated DCs at day 4 and 7 of culture compared to untreated DCs although this difference was not statistically significant (Figure 2.15A). Additionally, we observed that STAT5B was expressed at similar levels in ibrutinib-treated and untreated DCs during the course of development (Figure 2.15E). These results indicate that ibrutinib differentially regulates the expression of various STATs during the development of DCs.

We also evaluated the expression of various cytokines that are known to play a role in the development of myeloid cells like DCs. Upon treatment with ibrutinib, there was a trend towards higher levels of expression of IL-4, IL-6 and IL-13 at day 7 of culture (Figure 2.16A, 2.16B and 2.16C). Although levels of TNF-α were significantly lower in ibrutinib-treated DCs at day 4 of culture, ibrutinib-treated DCs showed
significantly higher levels of TNF-α compared to untreated DCs at day 7 of culture (Figure 2.16D). We observed that IL-12p35 subunit was significantly reduced upon ibrutinib treatment during the course of development (Figure 2.16E), while reduced levels of IL-12p40 were only observed at the earlier time point in ibrutinib-treated DCs (Figure 2.16F). Additionally, there was a trend towards reduced IL-10 and TGF-β levels upon ibrutinib treatment at the later time point (Figure 2.16G and 2.16H). We did not find any significant differences in IL-1β levels between ibrutinib-treated and untreated DCs (Figure 2.16I).

Other transcription factors known to participate and regulate DC development include PU.1, Flt3 and spiB (Carotta et al., 2010; Moore & Anderson, 2013). While PU.1 was induced at significantly higher levels at day 4 and 7 of culture in ibrutinib-treated DCs compared to untreated DCs, Flt3 was significantly upregulated in ibrutinib-treated DCs at the earlier time point (Figure 2.17A and 2.17B). SpiB was downregulated upon ibrutinib treatment at day 4 of culture but at day 7 there were no differences between ibrutinib-treated and untreated DCs (Figure 2.17C). Taken together, our results indicate that ibrutinib regulates the expression of various transcription factors and cytokines involved in the development of DCs.

Discussion

Ibrutinib treatment has a dual effect on DCs by modulating TLR-4 responses as well as DC development.

Our results indicate that LPS-mediated production of cytokines and NO upon ibrutinib treatment is dependent upon the development stage of cells as well as their
differentiation status at the time of treatment. LPS stimulation of ibrutinib-treated DCs, which were generated by treating bone marrow cells with ibrutinib during early stages of DC differentiation, led to an increase in the production of IFN-β with a concomitant decrease in the production of IL-6 and IL-12 compared to untreated DCs (Figure 2.7B, 2.7C and 2.7D). On the other hand when differentiated DCs were subject to LPS/ibrutinib treatment, there was an increase in the production of IL-6 and a decrease in the production of TNF-α (Figure 2.11A and 2.11D). Additionally while LPS/ibrutinib treatment in developing DCs led to an increase in MHC-II expression (Figure 2.4), there was a decrease in MHC-II expression when differentiated DCs were subject to LPS/ibrutinib treatment (Figure 2.12A and 2.12B). These contrasting differences between the two studies suggest that BTK plays a dual role in TLR-4 signaling as well as in the development of DCs.

We also observed that developing DCs treated with ibrutinib display higher levels of CD80 (Figure 2.2) compared to untreated DCs. Furthermore in the same study, LPS/ibrutinib-treated DCs showed higher levels of CD80 (Figure 2.4) and promoted higher rates of CD4⁺ T cell proliferation and T cell derived cytokines compared to LPS-only treated DCs (Figure 2.8A - 2.8D). A previous report by Kawakami et al. shows that bone marrow derived DCs cultured from BTK deficient mice display higher expression of MHC-II and CD80 compared to DCs from wildtype (WT) mice. Further upon LPS stimulation, BTK−/− DCs displayed higher levels of MHC-II and CD80 and promoted CD4⁺ T cell responses compared to WT DCs (Kawakami et al., 2006). These studies indicate that, in addition to the ability of BTK to modulate TLR-4 responses, BTK deficiency promotes the development of DCs, enhancing their activation status compared
to wildtype DCs. This study provides evidence for an inhibitory role for BTK during *in vitro* development of DCs which concurs with our own observations.

Our observations for higher IL-10 production and lower IL-12 production upon ibrutinib treatment in developing DCs (Figure 2.7A and 2.7D) is consistent with a recent study on LPS-mediated cytokine production in BTK<sup>−/−</sup> macrophages and DCs (Ní Gabhann et al., 2012). Further, the deficient nitric oxide production in both developing and differentiated DCs following ibrutinib treatment (Figure 2.7E and 2.11C) is consistent with previous reports on lower RNS and ROS production by LPS-stimulated macrophages from XID mice which possess a mutation in BTK that interferes with normal BTK signaling (Mangla et al., 2004; S. Mukhopadhyay et al., 2002). This decrease in NO production has been attributed to a lower induction of transcription factors, STAT-1 and IRF-1 in XID macrophages (Sangita Mukhopadhyay, George, & Bal, 1999). However, we observed that ibrutinib treatment in developing DCs does not alter the expression of STAT-1 and IRF-1 (Figure 2.14C and 2.15A) and therefore, indicating that NO production in ibrutinib-treated DCs is regulated in a STAT-1 and IRF-1-independent manner.

Although BTK deficient mouse models are useful to contextualize the combined effects of ibrutinib on DC development and TLR-4 responses, they do not allow us to probe how ibrutinib modulates TLR-4 responses alone, independent of its effects on the DC development. Studies that research the effects of BTK inhibitors on immune cells are more relevant and are potentially informative about the independent effects of ibrutinib on TLR-4 signaling. In this respect, there are no studies with BTK inhibitors that can allow us adequately discern this dual role for BTK. Ours is the first *in vitro* study
highlighting the cytokine responses of LPS-stimulated murine DCs upon treatment with BTK inhibitors. Hence, future studies should employ selective BTK inhibitors as a tool to better evaluate the role of BTK in myeloid cells.

**Ibrutinib enhances the maturation of DCs and their ability to activate T helper cell responses.**

Ibrutinib-treated DCs display characteristics of mature DCs. As mentioned before, our results show that ibrutinib promotes the expression of MHC-II and co-stimulatory molecule CD80 in developing DCs (Figure 2.2 and 2.4) which is supported by a report showing similar results in BTK−/− DCs (Kawakami et al., 2006). Ibrutinib-treated DCs were also less efficient at performing phagocytosis (Figure 2.3) which is yet another feature of mature DCs (Banchereau et al., 2000; Mellman & Steinman, 2001). Immature DCs express various receptors including C-type lectin receptors and Fcγ receptors which are downregulated during the transition of these cells into mature antigen-presenting cells (Banchereau et al., 2000). A previous report shows that BTK participates in FcγR-mediated phagocytosis, a critical process for the uptake of opsonized antigens (Jongstra-Bilen et al., 2008). However, the role of BTK in other forms of receptor-mediated phagocytosis has not been sufficiently explored. It is possible that ibrutinib may interfere with the ability of DCs to perform FcγR-mediated phagocytosis. However, based on previous studies that demonstrate the role of receptors such as C-type lectin receptors in the uptake of unopsonized *Leishmania* parasites (Colmenares, Corbi, Turco, & Rivas, 2004), the phagocytosis of *L. donovani* by DCs in our study is also likely to be mediated by such receptors rather than Fcγ receptors. Therefore, the decrease in the ability of
ibrutinib-treated DCs to phagocytose *Leishmania* parasites is likely a consequence of the higher maturation status of ibrutinib-treated DCs.

The maturation of DCs is associated with a downregulation of chemokine receptors expressed by immature DCs and upregulation of chemokine receptors expressed by mature DCs (McColl, 2002). We compared the expression of chemokine receptors which are typically expressed by immature DCs such as CCR2, CCR5, CCR6 and CXCR2. We observed that there was a four-fold decrease in the expression of CCR6 in ibrutinib-treated DCs at early time point of culture which switched to an approximately two-fold increase in the expression of this gene at a later time point (Figure 2.5C). Further, there was approximately three-fold decrease in the expression of CXCR2 in ibrutinib-treated DCs at a later time point of the culture (Figure 2.5D). We also observed that ibrutinib treatment in DCs enhanced the surface expression of CCR7 following LPS stimulation (Figure 2.6A) and this was associated with an increase in the number of ibrutinib-treated DCs migrating to the lymph nodes following injection into mouse footpads (Figure 2.6B). These results indicate that ibrutinib enhances the transition of immature to mature DCs by regulating the expression of various chemokines that are expressed at different stages of maturation.

Ibrutinib also enhanced the ability of DCs to activate CD4$^+$ T cell responses (Figure 2.8) which concurs with a previous study showing that BTK$^{-/-}$ DCs promote higher rates of T cell activation. This study also reported that BTK$^{-/-}$ DCs produce lower amounts of IL-10 compared to WT DCs and proposed that this decrease in IL-10 production was the mechanism underlying the increase in T cell proliferation (Kawakami et al., 2006). Interestingly, our results indicate that higher IL-10 production by DCs
following BTK inhibition is not necessarily associated with a reduction in DC-mediated T cell proliferation (Figure 2.7A and 2.8A). It is known that CD80/CD28 co-stimulation initiates the activation of naïve CD4⁺ T cells (Walsh & Mills, 2013) while IFN-β enhances the ability of DCs to induce T cell proliferation and cytokine production (Montoya et al., 2002). Hence, it is possible that the increased CD80 expression (Figure 2.4A and 2.4B) and IFN-β production (Figure 2.7B) upon ibrutinib treatment, which we observed in our study, compensate for the inhibitory effects of IL-10 on T cell activation and differentiation. On the other hand, there could be another mechanism by which ibrutinib-treated DCs enhance T cell proliferation. A study by Lu et al. demonstrated that deficient NO synthesis in DCs promotes DC-mediated T cell proliferation in vitro (Lu et al., 1996). This is possibly due to the ability of NO to disrupt the signaling pathway of cytokine IL-2 which is a key regulator of proliferation in T cells (Bingisser, Tilbrook, Holt, Bingisser, & Kees, 1998). We also observe a correlation between lower NO production by ibrutinib-treated DCs and higher rates of DC-mediated T cell proliferation (Figure 2.7E, 2.8A, 2.11C and 2.13A) in our study. Hence, the deficient NO synthesis by DCs upon ibrutinib treatment could also be responsible for the enhanced DC-mediated T cell proliferation.

Our study also demonstrates that the presence of other immune cells such as B cells in the DC-T cell co-culture alters the cytokine profile induced upon ibrutinib treatment on DCs (Figure 2.8E, 2.8F and 2.8G). It is likely that cytokines produced by effector B cells in the co-culture modulate T cell responses in a manner similar to a previous study (Harris et al., 2000). We observed that the presence of B cells led to a reduction in the levels of IFN-γ by T cells cultured with ibrutinib-treated DCs compared
to T cells cultured with untreated DCs (Figure 2.8E). Since IL-10 production by B cells suppresses Th1 responses (Mosmann, 2000), we measured whether there were differences in IL-10 levels between ibrutinib-treated and untreated DC co-cultures. However, we did not detect IL-10 in the culture supernatants which may indicate that B cells regulate IFN-γ production by T cells cultured with ibrutinib-treated DCs in an IL-10-independent manner.

**Ibrutinib regulates the expression of transcription factors and cytokines that are involved in the development of DCs.**

We observed that ibrutinib treatment led to a three-fold increase in the expressions of Type I Interferons, IFN-α and IFN-β (Figure 2.14A and 2.14B). Further there was a significant, albeit small, increase of approximately 1.5-fold in the expression of IRF-1 and IRF-5 by ibrutinib-treated DCs at an early time point during *in vitro* culture (Figure 2.14C and 2.14E). On the other hand, we observed a two-fold increase in the expression of IRF-7 in ibrutinib-treated DCs at a later time point of culture (Figure 2.14F). These results suggest that IFN-α, IFN-β and IRF-7 may play a role in promoting the maturation of ibrutinib-treated DCs. Indeed, a study by Montoya et al. shows that the production of Type I IFNs by DCs is critical for their phenotypic and functional maturation from bone marrow precursors. DCs which do not express Type I IFN-receptor, and therefore cannot respond to Type I IFNs, show decreased expression of co-stimulatory molecules and a reduced ability to activate CD4⁺ T cell proliferation. Furthermore, DCs treated with IFN-α and IFN-β showed an increase in the expression of co-stimulatory molecules (Montoya et al., 2002). These findings support our observations
regarding the correlation between increased expression of IFN-α and IFN-β by ibrutinib-treated DCs during development and the higher maturation status of ibrutinib-treated DCs. A previous report has also shown that IRF3, IRF5 and IRF7 regulate the signaling of Type I IFNs in myeloid DCs (Lazear et al., 2013). Based on our observations, it is likely that IRF-7 regulates IFN-α/β signaling pathway in ibrutinib-treated DCs since its upregulation coincides with the increase in IFN-α and IFN-β expression in culture. On the other hand, IRF-5 is upregulated at an earlier stage of DC development suggesting that it may not be involved downstream of the IFN-α/β signaling cascade in ibrutinib-treated DCs. It is possible that IRF-5 and IRF-1 could be playing a minor role in the enhancing the production of IFN-α and IFN-β by ibrutinib-treated DCs.

Treatment with ibrutinib modulates the expression of various STATs. Ibrutinib treatment led to higher induction of STAT2 (1.75-fold, Figure 2.15B), STAT5A (2-fold, Figure 2.15D) and STAT6 (1.5-fold, Figure 2.15F) during the early stage of development but these differences were not observed at the later time point. Although there was a trend towards higher STAT1 expression in ibrutinib-treated DCs, these differences were not statistically significant (Figure 2.15A). Additionally, we observed that STAT5B was expressed at similar levels in ibrutinib-treated and untreated DCs during the course of development (Figure 2.15E). Ibrutinib treatment also enhanced the expression of cytokines such as IL-4, IL-6 and IL-13, although the differences were not statistically significant (Figure 2.16A - 2.16C). On the other hand, other cytokines like TNF-α and IL-12p35 were downregulated following ibrutinib treatment (Figure 2.16D and 2.16E). There was also a trend towards lower expression of IL-10 and TGF-β induction by ibrutinib-treated DCs (Figure 2.16G and 2.16H). Additionally, ibrutinib promoted the
expression of DC development genes such as Flt3 and PU.1 while inhibiting the expression of spiB (Figure 2.17). These results clearly demonstrate that ibrutinib treatment modulates the expression of transcription factors and cytokines involved in the development of DCs. Although, BTK has been demonstrated to participate in DC development, there are few reports on targets of BTK in DCs. The changes in mRNA levels upon ibrutinib treatment suggest that BTK may transcriptionally regulate the expression of these genes, possibly in a manner similar to its regulation of TNF-α mRNA in monocytes (Horwood et al., 2003). Future studies on the role of BTK in DC development should focus on elucidating whether the above genes are directly targeted by BTK at the transcriptional level and the molecular mechanism by which BTK regulates the expression of these genes.

In summary, our results show that ibrutinib enhances the maturation of DCs and their ability to promote the activation of CD4⁺ T cells. Our results also provide a rationale for exploring the use of ibrutinib in the development of DC-based therapies for treatment of diseases such as cancer.
Figure 2.1. Schematic illustration of the activation signals provided by dendritic cells in order to prime T cell activation and differentiation. In addition to presenting antigen to the T cell receptor via the major histocompatibility complex molecules (MHC-I or MHC-II), dendritic cells also express co-stimulatory molecules such as CD40, CD80 and CD86 which direct CD4\(^+\) T cells to differentiate into various T helper subsets or CD8\(^+\) T cells into cytotoxic T cells. This differentiation process is also aided by cytokines synthesized by dendritic cells such as IL-12, IL-10, IL-6, TNF-α, TGF-β, IFN-α, IFN-β etc.
Figure 2.2. Ibrutinib treatment enhances the development of CD11c+ DCs and modulates the expression of DC maturation markers. (A) Dot plots show the percentages of CD11c+ DCs in untreated and ibrutinib-treated DC cultures. Numbers denote mean ± S.E.M of duplicate percentage values. (B) Histograms show the expressions of Ly6C, MHC-II, CD80 and CD86 in CD11c+ DCs from untreated and ibrutinib-treated DC cultures. Numbers denote mean ± S.E.M of duplicate percentage values of cells expressing the respective surface molecule. (C) Mean fluorescence intensities of Ly6C, MHC-II, CD80 and CD86 expression in CD11c+ DCs from untreated and ibrutinib-treated DC cultures. The data are presented as mean ± S.E.M of duplicate MFI values. Bone marrow cells from C57BL/6 mice were cultured in the presence of GM-CSF for 7 days. At Day 1 cultures were treated with PBS or 1µM ibrutinib to generate untreated and ibrutinib-treated DCs respectively. At Day 7, cells were harvested from untreated and ibrutinib-treated DC cultures and were stained with fluorescently labelled antibodies for the respective surface markers to determine their expression by flow cytometry. The data presented are representative of three independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 2.2.

A

B

C

Ly6C

MHC-II

CD80

CD86

Ly6C

MHC-II

CD80

CD86

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Ibrutinib reduces the ability of DCs to phagocytose *Leishmania* parasites. Graph shows the percentages of untreated and ibrutinib-treated DCs infected with *Leishmania* parasites at 3, 6 and 24 hours post-infection. Numbers denote mean ± S.E.M of duplicate percentage values. Untreated and ibrutinib-treated DCs were infected with red fluorescent *Leishmania* parasites for 3, 6 and 24 hours. At the respective time points, cells were harvested and stained with fluorescently labelled antibody for CD11c prior to analyzing parasite uptake by flow cytometry. Percentage of infected cells was determined by gating on CD11c<sup>+</sup> Leishmania<sup>+</sup> DCs. The data presented are representative of two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 2.4. Ibrutinib differentially regulates the expression of MHC-II and co-stimulatory molecules in LPS-activated DCs. (A) Histograms show the expressions of MHC-II, CD80, CD86 and CD40 in CD11c⁺ DCs from LPS/untreated and LPS/ibrutinib-treated DCs. Numbers denote mean ± S.E.M of duplicate percentage values. (B) Mean fluorescence intensities of MHC-II, CD80, CD86 and CD40 on CD11c⁺ DCs from untreated and ibrutinib-treated DCs upon LPS stimulation. The data are presented as mean ± S.E.M of duplicate MFI values. Untreated and ibrutinib-treated DCs were treated with control (media) or LPS (1 μg/ml) for 24 hours. After 24 hours, cells were stained with fluorescently labelled antibody for the respective surface molecules and their expressions were determined by flow cytometry. Analyses were conducted by gating on CD11c⁺ DCs. The data presented are representative of three independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 2.5. Ibrutinib modulates the expression of chemokine receptors during the development of DCs. Induction of (A) CCR2, (B) CCR5, (C) CCR6, (D) CCR7, (E) CXCR2 and CXCR4 in untreated and ibrutinib treated DC cultures at day 4 and day 7 of culture. Bone marrow cells from C57BL/6 mice were cultured in the presence of GM-CSF for 7 days. At Day 1 cultures were treated with PBS or 1µM ibrutinib to generate untreated and ibrutinib treated DCs respectively. At Day 4 and 7, cells were treated with TRIzol reagent to isolate RNA from cells and mRNA levels of the respective chemokine receptors were measured by real time q-PCR. The data are presented as mean ± S.E.M of duplicate sample values and is representative of two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 2.6. Ibrutinib enhances the proportion of CCR7-expressing DCs and promotes migration of DCs in vivo. (A) Dot plots show the percentages of CCR7+ DCs in LPS/untreated and LPS/ibrutinib-treated DCs. Numbers denote mean ± S.E.M of duplicate percentage values. Untreated and ibrutinib-treated DCs were treated with LPS (1 μg/ml) for 24 hours. After 24 hours, cells were stained with fluorescently labelled antibody for the respective surface molecules and their expressions were determined by flow cytometry. Analyses were conducted by gating on CD11c+ DCs. (B) Dot plots show the numbers of CFSE stained LPS/untreated and LPS/ibrutinib treated DCs that migrated to the draining popliteal lymph nodes following injection in the footpads of C57BL/6 mice. Numbers denote mean ± S.E.M of individual sample values from three mice per group. LPS/untreated and LPS/ibrutinib-treated DCs were generated as above, stained with CFSE and injected subcutaneously into the footpads of C57BL/6 mice. After 72 hours, mice were sacrificed to harvest draining popliteal lymph nodes. Lymph node cells were stained with fluorescently labelled antibody for CD11c to determine the numbers of migrated DCs. Analyses were conducted by gating on CD11c+ CFSE+ DCs. The data presented are representative of two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 2.6.

A

LPS/untreated

38.7 ± 0.4 %

LPS/Ibrutinib

52.9 ± 0.6 %

B

LPS/untreated

220 ± 58

LPS/Ibrutinib

449 ± 90
Figure 2.7. Ibrutinib differentially regulates cytokine production and nitric oxide responses by LPS-treated DCs. (A) IL-10, (B) IFN-β, (C) IL-6, (D) IL-12, (E) NO and (F) TNF-α production by untreated and ibrutinib-treated DCs upon LPS stimulation. Untreated and ibrutinib-treated DCs were treated with control (media) or LPS (1 µg/ml). After 24 hours of LPS treatment, cytokine production was determined in the culture supernatants by ELISA. After 48 hours of LPS treatment, NO levels were determined in the culture supernatants by measuring nitrite concentrations using Griess assay. The data are presented as mean ± S.E.M of triplicate sample values from two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 2.8. Ibrutinib-treated DCs enhance T cell proliferation and production of T cell derived cytokines. (A) Analysis of T cell proliferation upon co-culture with untreated, ibrutinib-treated, LPS/untreated or LPS/ibrutinib-treated DCs. Untreated and ibrutinib-treated DCs were pulsed with OVA (10 μg/ml) for 2 hours prior to treatment with LPS (1 μg/ml) for 22 hours. After OVA/LPS stimulation, DCs were cultured in 1:4 ratio with CFSE-stained T cells enriched from spleens of OT-II mice for 4 or 6 days. At Day 4 and 6, cells from co-culture were stained with anti-CD4 antibody and T cell proliferation was measured by flow cytometry. Analyses were conducted by gating on CD4+ population. Production of cytokines (B) IFN-γ, (C) IL-17 and (D) IL-13 in co-culture experiments performed as mentioned in A. At Day 4 and 6 of co-culture, cell culture supernatants were collected and the respective cytokines were measured by ELISA. Production of cytokines (E) IFN-γ, (F) IL-17 and (G) IL-13 in DC, T cell and B cell co-culture experiments. OVA pulsed LPS/untreated and LPS/ibrutinib-treated DCs were cultured in 1:4 ratio each with B cell fraction (B), T cell fraction (T) or both (T+B) prepared by nylon wool enrichment from spleens of OT-II mice for 6 days and cytokines were measured in the culture supernatants. The data are presented as mean ± S.E.M of duplicate sample values and is representative of two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 2.8.

A. T cell proliferation

B. IFN-γ

C. IL-17

D. IL-13

E. IFN-γ

F. IL-17

G. IL-13
Figure 2.9. Treatment with ITK selective inhibitor, PRN694, decreases the proportion of CD11c+ DCs and inhibits the maturation of DCs. (A) Dot plots show the percentages of CD11c+ DCs in untreated and PRN694-treated DC cultures. Numbers denote mean ± S.E.M of duplicate percentage values. (B) Histograms show the expressions of Ly6C, MHC-II and CD80 in CD11c+ DCs from untreated and PRN694-treated DC cultures. Numbers denote mean ± S.E.M of duplicate percentage values of cells expressing the respective surface molecule. (C) Mean fluorescence intensities of Ly6C, MHC-II and CD80 expression in CD11c+ DCs from untreated and PRN694-treated DC cultures. The data are presented as mean ± S.E.M of duplicate MFI values. Bone marrow cells from C57BL/6 mice were cultured in the presence of GM-CSF for 7 days. At Day 1 cultures were treated with PBS or 0.2 µM PRN694 to generate untreated and PRN694-treated DCs respectively. At Day 7, cells were harvested from untreated and PRN694-treated DC cultures and were stained with fluorescently labelled antibodies for the respective surface markers to determine their expression by flow cytometry. The data presented are representative of two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 2.9.

A

Untreated

PRN694

CD11c

SSC

57.5 ± 0.3%

49.8 ± 0.2%

B

Isotype ctrl

Untreated

PRN694

Ly6C

MHC-II

CD80

35.6 ± 0.8%

40.4 ± 0.4%

71.5 ± 0.3%

43.2 ± 0.3%

21.95 ± 0.05%

40.65 ± 0.05%

C

Ly6C

MHC-II

CD80

**

*

**

*
Figure 2.10. Treatment with PRN694 does not affect cytokine production LPS-stimulated DCs and DC-mediated T cell responses. (A) IL-12, (B) IL-10, (C) IL-6 and (D) TNF-α production by untreated and PRN694-treated DCs upon LPS stimulation. Untreated and PRN694-treated DCs were treated with control (media) or LPS (1 μg/ml). After 24 hours of LPS treatment, cytokine production was determined in the culture supernatants by ELISA. The data are presented as mean ± S.E.M of triplicate sample values from two independent experiments. (E) Analysis of T cell proliferation upon co-culture with untreated, PRN694-treated, LPS/untreated or LPS/PRN694-treated DCs. Untreated and PRN694-treated DCs were pulsed with OVA (10 μg/ml) for 2 hours prior to treatment with LPS (1 μg/ml) for 22 hours. After OVA/LPS stimulation, DCs were cultured in 1:4 ratio with CFSE-stained T cells enriched from spleens of OT-II mice for 6 days. At Day 6, cells from co-culture were stained with anti-CD4 antibody and T cell proliferation was measured by flow cytometry. Analyses were conducted by gating on CD4⁺ T cells. The data are presented as mean ± S.E.M of duplicate percentage values and is representative of two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 2.11. Ibrutinib modulates cytokine and nitric oxide production in differentiated DCs upon LPS stimulation. (A) TNF-α, (B) IL-12 and (C) nitric oxide (NO) production in untreated and ibrutinib-treated DCs stimulated with LPS. DCs were generated by culturing bone marrow cells from C57BL/6 mice in the presence of GM-CSF for 7 days. At day 7, DCs were harvested and rested for 24 hours. Rested DCs were treated with DMSO or ibrutinib (1 µM), washed twice and treated with LPS (1 µg/ml). After 24 hours of LPS treatment, cytokine production was determined in the culture supernatants by ELISA. After 48 hours of LPS treatment, NO levels were determined in the culture supernatants by measuring nitrite concentrations by Griess assay. The data are presented as mean ± S.E.M of triplicate sample values from three independent experiments. (D) IL-6, (E) IL-18 and (F) TGF-β mRNA induction in untreated and ibrutinib-treated DCs upon LPS stimulation. Differentiated DCs were treated with DMSO/ibrutinib and LPS as mentioned above. After 18 hours of LPS treatment, cells were treated with TRIzol Reagent, RNA was isolated from cells and mRNA levels of respective cytokines were determined by real-time qPCR. The data are presented as mean ± S.E.M of duplicates obtained by pooling three independent samples and is representative of two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Ibrutinib regulates the surface expression of MHC-II and co-stimulatory molecules in differentiated DCs upon LPS stimulation. Histogram plots show expressions of (A) MHC-II, (B) CD86 and (C) CD80 in LPS/untreated and LPS/ibrutinib-treated DC cultures. Numbers represent mean percentage of cells ± S.E.M of the respective surface molecule on DCs. The data presented are representative plots of three independent experiments. Mean fluorescence intensities (MFIs) of (D) MHC-II, (E) CD86 and (F) CD80 expression in LPS/untreated and LPS/ibrutinib-treated DC cultures. DCs were generated by culturing bone marrow cells from C57BL/6 mice in the presence of GM-CSF for 7 days. At day 7, DCs were harvested and rested for 24 hours. Restered DCs were treated with DMSO or ibrutinib (1 µM), washed twice and treated with LPS (1 µg/ml). After 24 hours of LPS treatment, cells were blocked, stained with conjugated antibodies for the respective surface molecules and expressions of the surface molecules were determined by flow cytometry. Analyses were conducted by gating on CD11c+ DCs. The data are presented as mean ± S.E.M of representative MFI values of three independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 2.13. Ibrutinib-treated DCs promote T cell proliferation and IL-17 production upon co-culture with T cells. (A) Analysis of T cell proliferation upon co-culture with untreated, ibrutinib-treated, LPS/untreated or LPS/ibrutinib-treated DCs. DCs were generated by culturing bone marrow cells from C57BL/6 mice in the presence of GM-CSF for 7 days. At day 7, DCs were harvested and rested for 24 hours. Rested DCs were treated with control (DMSO) or ibrutinib (1 µM), washed twice, pulsed with OVA (10 µg/ml) for 2 hours and treated with LPS (1 µg/ml) for 22 hours. After OVA/LPS stimulation, DCs were cultured in 1:4 ratio with CFSE-stained T cells enriched from spleens of OT-II mice for 6 days. At Day 6, cells from co-culture were blocked, stained with anti-CD4 antibody and T cell proliferation was measured by flow cytometry. Analyses were conducted by gating on CD4+ T cells. The data are presented as mean ± S.E.M of duplicate percentage values and is representative of two independent experiments. (B) Production of T cell cytokines IL-17, (C) IFN-γ and (D) IL-13 in co-culture experiments performed as mentioned in A. At Day 6 of co-culture, cell culture supernatants were collected and the respective cytokines were measured by ELISA. The data are presented as mean ± S.E.M of duplicate sample values and is representative of two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 2.14. Ibrutinib upregulates the induction of Type I interferons and IFN-associated transcription factors during the development of DCs. Induction of (A) IFN-α, (B) IFN-β, (C) IRF-1, (D) IRF-3, (E) IRF-5 and (F) IRF-7 in untreated and ibrutinib-treated DC cultures at day 4 and day 7 of culture. Bone marrow cells from C57BL/6 mice were cultured in the presence of GM-CSF for 7 days. At Day 1 cultures were treated with PBS or 1µM ibrutinib to generate untreated and ibrutinib-treated DCs respectively. At Day 4 and 7, cells were treated with TRIzol reagent to isolate RNA from cells and mRNA levels of the respective cytokines and transcription factors were measured by real time q-PCR. The data are presented as mean ± S.E.M of duplicate sample values and is representative of two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 2.15. Treatment with ibrutinib modulates the expression of STATs during DC development of DCs. Induction of (A) STAT1, (B) STAT2, (C) STAT3, (D) STAT5A, (E) STAT5B and (F) STAT6 in untreated and ibrutinib-treated DC cultures at day 4 and day 7 of culture. Bone marrow cells from C57BL/6 mice were cultured in the presence of GM-CSF for 7 days. At Day 1 cultures were treated with PBS or 1µM ibrutinib to generate untreated and ibrutinib-treated DCs respectively. At Day 4 and 7, cells were treated with TRIzol reagent to isolate RNA from cells and mRNA levels of the respective transcription factors were measured by real time q-PCR. The data are presented as mean ± S.E.M of duplicate sample values and is representative of two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 2.16. Ibrutinib differentially regulates the expression of cytokines involved in the development of DCs. Induction of (A) IL-4, (B) IL-6, (C) IL-13, (D) TNF-α, (E) IL-12p35, (F) IL-12p40, (G) IL-10, (H) TGF-β and (I) IL-1β in untreated and ibrutinib-treated DC cultures at day 4 and day 7 of culture. Bone marrow cells from C57BL/6 mice were cultured in the presence of GM-CSF for 7 days. At Day 1 cultures were treated with PBS or 1µM ibrutinib to generate untreated and ibrutinib-treated DCs respectively. At Day 4 and 7, cells were treated with TRIzol reagent to isolate RNA from cells and mRNA levels of the respective cytokines were measured by real time q-PCR. The data are presented as mean ± S.E.M of duplicate sample values and is representative of two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 2.16.

- **A** IL-4
- **B** IL-6
- **C** IL-13
- **D** TNF-α
- **E** IL-12p35
- **F** IL-12p40
- **G** IL-10
- **H** TGF-β
- **I** IL-1β

Fold change for each condition over days 4 and 7.
Figure 2.17. Ibrutinib regulates the expression of transcription factors involved in DC development. Induction of (A) PU.1, (B) Flt3 and (C) spiB in untreated and ibrutinib-treated DC cultures at day 4 and day 7 of culture. Bone marrow cells from C57BL/6 mice were cultured in the presence of GM-CSF for 7 days. At Day 1 cultures were treated with PBS or 1µM ibrutinib to generate untreated and ibrutinib-treated DCs respectively. At Day 4 and 7, cells were treated with TRIzol reagent to isolate RNA from cells and mRNA levels of the respective transcription factors were measured by real time q-PCR. The data are presented as mean + S.E.M of duplicate sample values and is representative of two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
CHAPTER 3: IBRUTINIB DAMPENS A TH2 IMMUNE RESPONSE AND INCREASES RESISTANCE TO CUTANEOUS LEISHMANIASIS.

Abstract

Cutaneous leishmaniasis (CL) is a vector-borne parasitic disease caused by different organisms within the genus *Leishmania*, including *Leishmania major*. CL typically manifests in humans as a localized lesion near the bite of the sandfly vector which may depending upon the infecting species and the immune status of the patient. It is known that B cells participate in the immunopathogenesis of this disease and contribute to susceptibility during infection. Since ibrutinib inhibits BTK, an enzyme critical for B cell development and function, we evaluated whether ibrutinib would inhibit B cell function during CL and thereby, be effective in controlling this disease. We studied this by employing a murine footpad model of infection where vehicle or ibrutinib was administered via drinking water to mice from the day of infection. We observed that ibrutinib treatment led to smaller lesions and lower parasitic loads at the infection site. Further, there was a significant reduction in the production of Th2-associated cytokines such as IL-4, IL-10 and IL-13 in ibrutinib-treated mice compared to vehicle-treated mice, but no difference in Th1 cytokine IFN-γ between the two groups. Further, there was a decrease in the levels of Th2-associated antibody, IgG1, following ibrutinib treatment while there was no difference in Th1-associated antibody, IgG2a, between vehicle-treated and ibrutinib-treated mice. In addition to targeting BTK, ibrutinib also inhibits ITK which
is critical for the function of Th2 cells. In order to study whether the dampening of Th2 response by ibrutinib is mediated by the inhibition of ITK activity, we evaluated the progression of CL following treatment with an ITK selective inhibitor, PRN694. We observed that PRN694 treatment had no effect on the course of infection by *L. major*. Further, we did not observe any differences in Th1 or Th2 cytokines between vehicle-treated and PRN694-treated mice upon infection with *L. major*. These results suggest that the decrease in Th2 responses upon ibrutinib treatment in *L. major* infected mice may not be dependent on the inhibition of ITK. Future studies should focus on the mechanism by which ibrutinib mediates resistance to CL. Taken together, our results indicate that ibrutinib is effective in inhibiting the progression of CL and should be further explored as an immunotherapeutic candidate for treating this disease.

**Introduction**

Leishmaniasis, a neglected tropical disease which affects over 12 million people worldwide, is caused by a protozoan parasite called *Leishmania* and is a vector-borne disease which is transmitted by parasite infected sandflies to mammalian hosts (Alvar et al., 2012; McGwire & Satoskar, 2014). After transmission, infected individuals can remain asymptomatic or can display diverse clinical manifestations depending on the infective species and the patients’ immunological status. Based on these factors, there are two major manifestations of the disease: cutaneous leishmaniasis and visceral leishmaniasis. Cutaneous leishmaniasis (CL) is characterized by lesions proximal to the site of the sandfly bite which may resolve or spread depending on the immune status of the host. During visceral leishmaniasis (VL), the parasite disseminates to the visceral organs like spleen, liver and bone marrow leading to severe immunopathology which is
often fatal to the host. CL is caused by various Leishmania species which are widely distributed in countries of Asia, Africa and South America. The annual incidence of CL is 0.7-1.2 million and 70-75% of global cases occur in Afghanistan, Algeria, Columbia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rice and Peru (Oghumu, Natarajan, & Satoskar, 2015). Leishmania major is one of the major causative organisms of CL in the Old World and will be a focus of this chapter (Alvar et al., 2012; McGwire & Satoskar, 2014; Oghumu et al., 2015).

Immunity to leishmaniasis is mediated by the activation of a strong innate and adaptive immune response. During the early phase of the disease when the parasites are inoculated into the skin, innate immune cells such as macrophages and dendritic cells play a critical role in initiating an inflammatory response. Dendritic cells produce IL-12 to activate a protective adaptive immune response by priming CD4+ T cells into IFN-γ-producing Th1 cells. IFN-γ subsequently activates macrophages, which are the eventual hosts of the parasite, to produce leishmanicidal molecules like nitric oxide which control the infection (Lopes, Costa-Da-Silva, & Dosreis, 2014; Oghumu et al., 2015). On the other hand, the production of IL-4 and IL-13 by Th2 cells contributes to disease progression. These cytokines favor the alternative activation of macrophages which promotes parasite growth and replication within macrophages and the establishment of chronic infection (Hölscher, Arendse, Schwegmann, Myburgh, & Brombacher, 2006; McMahon-Pratt & Alexander, 2004; Oghumu et al., 2015). Additionally, the synthesis of IL-10 by macrophages and regulatory T cells dampens protective immune responses to enhance the persistence of parasites (McMahon-Pratt & Alexander, 2004; Sacks & Noben-Trauth, 2002).
Besides Th2 cells and alternatively activated macrophages, B cells also mediate susceptibility to *L. major* infection. It is known that XID mice, which express an inactivated BTK leading to defective B cell development and therefore lower peripheral B cell counts, are more resistant to *L. major* compared to controls. Further, treatment of these mice with IL-7 which enhances B cell counts restored susceptibility to *L. major* infection (Babai, Louzir, Cazenave, & Dellagi, 1999; Hoerauf, Solbach, Rollinghoff, & Gessner, 1995). These results indicate that reduction in the numbers and activity of B cells leads to an increase in resistance to *L. major* infection. Since ibrutinib inhibits B cell activity, we hypothesized that treatment with ibrutinib would curtail the progression of *L. major* infection. We decided to study this using a murine footpad infection model of *L. major* in susceptible BALB/c mouse strain.

**Materials and Methods**

**Mice strains.** Female BALB/c mice (age 8-10 weeks) were purchased from Harlan Laboratories. All animals were housed in a pathogen-free facility in The Ohio State University in accordance with National Institutes of Health and institutional guidelines.

**Parasite cultures and infections.** *Leishmania major* (strain LV39) were maintained by serial passages in BALB/c mice infected with parasites in the left footpad. For culturing parasites for *in vivo* studies, infected BALB/c mice were sacrificed to harvest footpad lesions containing virulent parasites. Single cell suspensions were prepared from the lesions and cultured in complete M199 medium (M199 medium (Gibco) supplemented with 10% fetal bovine serum (Atlanta Biologicals), 1% penicillin (20
Units/ml)/streptomycin (20 µg/ml) (Life Technologies) and 1% HEPES (Gibco)) to generate promastigotes. Parasites were serially passaged in complete M199 medium for approximately 2 weeks to generate stationary-phase promastigotes (in P4 - P6 passage) for infections. The infections were conducted by subcutaneously injecting 2 million *L. major* parasites in the rear left footpads of female BALB/c mice (age 8-10 weeks). For ibrutinib treatment studies, vehicle (DMSO) or ibrutinib (1 µM) was administered to infected mice via drinking water at the start of the infection until the end of the experiment. For PRN694 treatment studies, vehicle (DMSO) or PRN694 (0.2 µM) was administered to infected mice once daily via oral gavage from the day of the infection until the end of the experiment. Lesions sizes were monitored with a dial-gauge micrometer at weekly intervals up to 9 weeks of infection or until the mice reach the maximum lesion size under the IACUC protocol. At regular intervals during the course of infection, mice were sacrificed to analyze parasite burdens and immune responses generated during infection. At week 9 post-infection in ibrutinib treatment studies, blood was collected from infected mice by tail-vein bleeding and centrifuged at 200 x g to isolate sera which was analyzed for the levels of *Leishmania*-specific antibodies by ELISA (Stamm et al., 1998).

**Quantitation of parasite burdens.** In ibrutinib treatment studies, *L. major* infected vehicle-treated and ibrutinib-treated mice were sacrificed at week 6 and 9 post-infection for analysis of parasite burdens. In PRN694 treatment studies, *L. major* infected vehicle-treated and PRN694-treated mice were sacrificed at week 2 and 6 post-infection for analysis of parasite burdens. At the respective time points, infected footpads were excised
and parasite burden was determined as described before (Satoskar, Okano, & David, 1997). The results were expressed in log parasite dilution.

**T cell proliferation assay.** At the respective time points, draining popliteal lymph nodes were excised from infected mice and T cell proliferation assay was performed as described previously (Satoskar et al., 1997). Briefly, single cell suspensions were prepared from lymph nodes and 3 X 10^5 lymph node cells were plated in 96-well flat-bottomed plates and stimulated with 20 µg/ml freeze-thaw prepared *L. major* antigen for 72 hours at 37°C. T cell proliferation was evaluated by alamarBlue reduction method. Briefly, at 60 hours post-stimulation with *L. major* antigen, cells were incubated with 10% alamar blue (Life Technologies) for 12 hours prior to measuring the absorbance at 570nm and 600nm using Spectramax Microplate reader and Softmax Pro software (Molecular Devices LLC). Absorbance values were used to calculate the rate of alamarBlue reduction and therefore, T cell proliferation using online alamarBlue colorimetric calculator (AbD Serotec). After 72 hours of stimulation with *L. major* antigen, culture supernatants were collected to analyze the production of cytokines such as IFN-γ, IL-4, IL-10, IL-13 and IL-17 by ELISA (Oghumu et al., 2014).

**Cytokine ELISA.** Cytokine ELISA was performed as described previously with some modifications (Oghumu et al., 2014). Anti-mouse capture and biotinylated detection antibodies for IL-10, IL-17 (Biolegend), IL-13 (eBiosciences), IFN-γ and IL-4 (BD Biosciences) were used for the respective ELISA. Recombinant mouse IFN-γ, IL-4, IL-10 and IL-13 (BD Biosciences) and IL-17 (eBiosciences) were used as standards.
Detection of cytokines was performed using streptavidin conjugated alkaline phosphatase (BD Pharmingen) and p-nitrophenyl phosphate (PNPP) tablets (Thermo Fisher Scientific) as substrate. Plates were read using Spectramax M3 microplate reader (Molecular Devices LLC) at an absorbance of 405 nm. Cytokine concentrations were determined by extrapolation from the generated standard curve using Softmax Pro software (Molecular Devices LLC).

**Antibody ELISA.** Antibody ELISA was performed to analyze the levels of Th1 and Th2-associated antibodies as described previously (Stamm et al., 1998). Anti-mouse primary and secondary HRP-conjugate antibodies for IgG1 and IgG2a (Biolegend) were used for the respective ELISA. Detection of antibodies was performed using TMB (Thermo Fisher Scientific) as a substrate and 5% phosphoric acid as a stopping solution. Plates were read using Spectramax M3 microplate reader (Molecular Devices LLC) at an absorbance of 450 nm and antibody titers were determined using Softmax Pro software (Molecular Devices LLC).

**Statistical analysis.** All statistical analyses were done using Prism 5 (GraphPad Software). Student’s unpaired *t* test was employed to determine statistical significance of values obtained. The *p* values less than 0.05 were considered statistically significant.

**Results**

We studied the efficacy of ibrutinib in treating a mouse footpad model of *L. major* infection by comparing the lesion sizes in *L. major* infected mice treated with either
vehicle or ibrutinib from the start of the infection (Figure 3.1). Both vehicle-treated and ibrutinib-treated mice showed similar lesion sizes until 6 weeks post-infection. After 6 weeks, ibrutinib-treated mice displayed smaller lesions compared to vehicle-treated mice (Figure 3.2A). In order to evaluate whether the differences in parasite burdens correlated with lesion sizes, we harvested the mice at weeks 6 and 9 post-infection to measure parasite loads in the infected footpads. At week 6, there was no significant difference in parasite burdens between vehicle-treated and ibrutinib-treated mice. On the other hand, at week 9, ibrutinib-treated mice had significantly lower parasite burden (approximately 10 fold) compared to vehicle-treated mice (Figure 3.2B). Therefore, the difference in parasite burdens at week 6 and 9 correlate with difference in lesion sizes in vehicle-treated and ibrutinib-treated mice.

We studied how ibrutinib modulated anti-leishmania immune responses evaluating T cell proliferation and cytokine responses generated by the cells of the draining lymph node following stimulation with L. major antigen. At week 6 post-infection, T cell proliferation was similar between vehicle-treated and ibrutinib-treated mice (Figure 3.3A). Although IFN-γ production was significantly lower in ibrutinib-treated mice compared to vehicle-treated mice, there were no differences in IL-4 and IL-10 levels between the two groups (Figure 3.3B – 3.3D). At week 9 post-infection, we observed that there were no significant differences in T cell proliferation and IFN-γ production between vehicle-treated and ibrutinib-treated mice (Figure 3.3E and 3.3F). Interestingly, ibrutinib-treated mice showed significant reduction in the levels of IL-4, IL-10 and IL-13 compared to vehicle-treated mice (Figure 3.3G – 3.3I). When we measured the ratios of Th1/Th2 cytokines at week 9 post-infection, we observed that
ibrutinib-treated mice showed higher ratios of IFN-γ/IL-4, IFN-γ/IL-10 and IFN-γ/IL-13 compared to vehicle-treated mice (Figure 3.4A – 3.4C). These results indicate that ibrutinib-treated mice display a Th1-skewed cytokine profile which could be contributing to resistance against *L. major* at later stages of infection. We also evaluated the Th1- and Th2-associated antibodies in the sera of vehicle-treated and ibrutinib-treated mice at week 9 post-infection. We observed that Th2-associated antibody, IgG1, was lower in ibrutinib-treated mice while there were similar levels of Th1-associated antibody, IgG2a (Figure 3.5). These results show that the antibody responses in ibrutinib-treated mice are skewed towards Th1-associated immune response, similar to the cytokine profile observed in these mice.

It is known that ibrutinib targets both BTK and ITK among the TEC family kinases (Burger & Buggy, 2013; Dubovsky et al., 2013). Since the Th1-skewed response observed in our study could be mediated by ITK inhibition, we hypothesized that a similar immunological profile will be observed when *L. major* infected mice were treated with an ITK selective inhibitor such as PRN694 (Dubovsky et al., 2013). Hence, we studied the progression of *L. major* infection in mice that were treated with either vehicle or PRN694 at the time of infection (Figure 3.6). Surprisingly, we observed that lesion sizes and the progression of the disease were similar between vehicle-treated and PRN694-treated mice (Figure 3.7A). The lesion size data in vehicle-treated and PRN694-treated mice was corroborated by comparable parasite burdens between these two groups of mice at week 2 and week 6 post-infection (Figure 3.7B). Additionally, we did not find any differences in T cell proliferation or cytokine production between vehicle-treated and PRN694-treated mice at both weeks 2 and 6 post-infection (Figure 3.8). These results
indicate that inhibition of ITK does not promote Th1 skewed immune response as we had hypothesized previously. Hence, it is likely that ibrutinib curtails the progression of \textit{L. major} infection in an ITK-independent manner.

**Discussion**

Our results show that ibrutinib suppressed the production of Th2 cytokines but does not alter the synthesis of Th1 cytokines during \textit{L. major} infection (Figure 3.3). We observed the same trend when we measured the levels of Th1- and Th2-associated antibodies following ibrutinib treatment in \textit{L. major} infected mice. Ibrutinib dampened the levels of IgG1, a Th2-associated antibody, but does not affect the levels of IgG2a, a Th1-associated antibody (Figure 3.5). Our hypothesis was that ibrutinib inhibits the progression of \textit{L. major} infection by dampening the function of B cells. If our hypothesis was correct, we would have observed a reduction in antibody production in ibrutinib-treated mice leading to lower levels of both Th1- and Th2-associated antibodies in these mice compared to vehicle-treated mice, which is contrary to our results (Figure 3.5). On the other hand, our results suggest that ibrutinib acts on T helper cells to selectively dampen Th2 responses without altering Th1 responses and thereby, promotes an overall Th1-skewed immune response during CL. However, the previously described role for ibrutinib in inhibiting BTK and thereby, B cell function does not explain how ibrutinib alters the Th1/Th2 cytokine profile during \textit{L. major} infection. This suppression of Th2 cytokines could be attributed to the blocking of ITK activity by ibrutinib, specifically in Th2 cells where this TEC kinase is critical for cellular function (Dubovsky et al., 2013). In this respect, it has been previously demonstrated that ITK-deficient CD4+ T cells fail to produce IL-4 under Th2 priming conditions \textit{in vitro}. Further, ITK-deficient mice
showed impairments in the secretion of Th2 cytokines while the production of Th1 cytokine was not affected in these mice (Fowell et al., 1999). Based on these reports, we further investigated the mechanism by which ibrutinib could be curtailing the progression of CL by using an ITK selective inhibitor, PRN694, to study whether inhibition of ITK activity alone dampens Th2 cytokines and thereby mediates resistance to *L. major* infection. Surprisingly, we observed that treatment with PRN694 had no effect on the course of lesion development in CL (Figure 3.7). Further, there were no significant differences in T cell proliferation or cytokine production between vehicle-treated and PRN694-treated mice following infection with *L. major* (Figure 3.8). These results indicate the inhibition of ITK activity alone does not promote protection to *L. major* infection. Hence, it is possible that ibrutinib mediates resistance to CL through the inhibition of BTK alone or through a combined inhibition of both BTK and ITK.

It is also possible that the Th1-skewed cytokine profile observed in ibrutinib-treated mice occurs via an alternate mechanism wherein ibrutinib targets other immune cells, such as antigen-presenting cells, involved in T helper cell differentiation to inhibit Th2 cell development and in this manner, decreases the secretion of Th2 cytokines. It is known that dendritic cells (DCs) are important for the development of protective immune response during CL. DCs such as Langerhans cells are located in the skin where they ingest *Leishmania* antigen at the site of infection. They participate in the development of Th1 response by upregulating MHC-II and co-stimulatory molecules and releasing IL-12 following the uptake of *L. major* parasites (von Stebut, Belkaid, Jakob, Sacks, & Udey, 1998). Results from our previous chapter indicated that ibrutinib treatment led to an increase in the maturation of DCs, enhancing their ability to prime CD4^+^ T cell
responses. Specifically, ibrutinib modulates the expression of co-stimulatory molecules on DCs which are involved in mediating the differentiation of various T helper cell subsets. While ibrutinib treatment on DCs increases the expression of CD80, a Th1 associated co-stimulatory molecule, it reduces the expression of CD86, a Th2 associated co-stimulatory molecule (Figure 2.2 and 2.4). Therefore, it is possible that ibrutinib regulates the expression of these co-stimulatory molecules on DCs to differentially prime T helper responses during infection with L. major. Besides DCs, other immune cells also act as antigen-presenting cells during L. major infection. Interestingly, a report highlighted the antigen-presentation function of B cells as the mechanism by which these cells contribute to susceptibility during L. major infection rather than antibody effector function. The study by Ronet et al. shows that B cell deficient mice infected with L. major display a decrease in IL-4 and IL-10 levels compared to wildtype controls while IFN-γ levels are similar between both groups of mice. Further, only adoptive transfer of wildtype B cells and not immune serum transfer restored susceptibility to L. major infection in these mice (Ronet et al., 2008). These results demonstrate the role that B cells play in promoting Th2 response during L. major infection, likely due to their antigen-presentation function. This observation regarding the antigen-presentation role of B cells during Leishmania infection has been corroborated by a report in humans where B cells from Leishmania infected patients enhance the activation of CD4+ T cells. Further, this B cell-mediated activation of T cells was associated with the upregulation of CD86 expression on B cells (Rodriguez-Pinto, Saravia, & McMahon-Pratt, 2014). Although it has been shown that the expression of CD86 on B cells is regulated in a BTK-dependent manner following stimulation with IgM and TLR9 ligand (Kenny et al., 2013), whether
BTK also regulates B cell-specific expression of CD86 and its importance in B cell-mediated Th2 cell development during *Leishmania* infection has not been studied thus far. Taken together, our studies provide a rationale for the use of ibrutinib in the treatment of cutaneous leishmaniasis. Further, ibrutinib should be further explored as a potential drug candidate in treating other infectious diseases, particularly neglected tropical diseases.
Figure 3.1. Experimental design for study evaluating the effect of ibrutinib on *Leishmania major* infection. Female BALB/c mice (age 8-10 weeks) were subcutaneously injected with 2 million stationary-phase *Leishmania major* promastigotes in the hind footpad. After infection, mice were separated into two groups (n=20 each) for vehicle or ibrutinib treatment and were administered with vehicle or ibrutinib via drinking water on the day of infection. Lesion sizes were monitored on a weekly basis with a dial-gauge micrometer until 9 weeks post-infection. At weeks 6 and 9 post-infection, infected mice were sacrificed to analyze parasite burdens, T cell proliferation and cytokine production in vehicle-treated and ibrutinib-treated mice.
Figure 3.2. Treatment with ibrutinib reduces lesion development and parasite burden during the course of *L. major* infection. (A) Graph showing size of lesions in vehicle-treated and ibrutinib-treated mice infected with *Leishmania major* during 9 weeks of infection. The data are presented as mean ± S.E.M of lesion size measurements in total of n=20 mice per group. (B) Graphs show parasite burdens in *L. major* infected vehicle-treated and ibrutinib-treated mice at weeks 6 and 9 post-infection. The data are presented as mean ± S.E.M of log parasite dilution values in total of n=10 mice per group. Female BALB/c mice (age 8-10 weeks) were subcutaneously injected with 2 million stationary-phase *Leishmania major* promastigotes in the hind footpad. On the same day, infected mice were treated with vehicle or ibrutinib administered *via* drinking water. Lesion sizes were monitored on a weekly basis with a dial-gauge micrometer until 9 weeks post-infection. At weeks 6 and 9 post-infection, infected mice were sacrificed to analyze parasite burden by limiting dilution assay. The data are representative of two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 3.2.

(A) Lesion size

(B) Parasite burden

Week 6  Week 9

N.S.

Vehicle  ibrutinib

Vehicle  ibrutinib
Figure 3.3. Treatment with ibrutinib inhibits the production of IL-4, IL-10 and IL-13 during *L. major* infection. (A) T cell proliferation, (B) IFN-γ, (C) IL-4 and (D) IL-10 production by *L. major* infected vehicle-treated and ibrutinib-treated mice at week 6 post-infection. (E) T cell proliferation, (F) IFN-γ, (G) IL-4, (H) IL-10 and (I) IL-13 production by *L. major* infected vehicle-treated and ibrutinib-treated mice at week 9 post-infection. The data are presented as mean + S.E.M of sample values from n=10 mice per group. Female BALB/c mice (age 8-10 weeks) were subcutaneously injected with 2 million stationary-phase *Leishmania major* promastigotes in the hind footpad. On the same day, infected mice were treated with vehicle or ibrutinib administered *via* drinking water. At weeks 6 and 9 post-infection, infected mice were sacrificed and draining popliteal lymph nodes were isolated to prepare single cell suspensions. Lymph node cells were stimulated with 20 µg/ml freeze-thaw prepared *L. major* antigen. After 60 hours of stimulation, T cell proliferation was determined by alamarBlue reduction method. After 72 hours of stimulation, culture supernatants were collected to determine the cytokine production by ELISA. The data are representative of two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 3.3.
Figure 3.4. Ibrutinib treatment promotes a Th1-skewed cytokine response during *L. major* infection. Ratios of (A) IFN-γ/IL-4, (B) IFN-γ/IL-10 and (C) IFN-γ/IL-13 in vehicle-treated and ibrutinib-treated mice infected with *L. major* at week 9 post-infection. The data are presented as mean ± S.E.M of sample values from n=10 mice per group. Female BALB/c mice (age 8-10 weeks) were subcutaneously injected with 2 million stationary-phase *Leishmania major* promastigotes in the hind footpad. On the same day, infected mice were treated with vehicle or ibrutinib administered via drinking water. At weeks 9 post-infection, infected mice were sacrificed and draining popliteal lymph nodes were isolated to prepare single cell suspensions. Lymph node cells were stimulated with 20 µg/ml freeze-thaw prepared *L. major* antigen for 72 hours. At 72 hours post-stimulation, culture supernatants were collected to determine the cytokine production by ELISA. The ratios were calculated by using the amounts of the respective cytokines from individual mice in vehicle-treated and ibrutinib-treated groups. The data are representative of two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 3.5. Ibrutinib treatment dampens the production of Th2-associated antibody, IgG1, but has no effect on Th1-associated antibody, IgG2a, during *L. major* infection. Antibody titers of (A) IgG1 and (B) IgG2a in vehicle-treated and ibrutinib-treated mice infected with *L. major* at week 9 post-infection. The data are presented as mean + S.E.M of sample values from n=10 mice per group. Female BALB/c mice (age 8-10 weeks) were subcutaneously injected with 2 million stationary-phase *Leishmania major* promastigotes in the hind footpad. On the same day, infected mice were treated with vehicle or ibrutinib administered *via* drinking water. At weeks 9 post-infection, blood was collected from infected mice to harvest sera and analyze the production of the respective antibodies by ELISA. The data are representative of two independent experiments.
Figure 3.6. Experimental design for study evaluating the effect of PRN694 on *Leishmania major* infection. Female BALB/c mice (age 8-10 weeks) were subcutaneously injected with 2 million stationary-phase *Leishmania major* promastigotes in the hind footpad. After infection, mice were separated into two groups (n=20 each) for vehicle or PRN694 treatment and were administered with vehicle or PRN694 via drinking water on the day of infection. Lesion sizes were monitored on a weekly basis with a dial-gauge micrometer until 6 weeks post-infection. At weeks 2 and 6 post-infection, infected mice were sacrificed to analyze parasite burdens, T cell proliferation and cytokine production in vehicle-treated and ibrutinib-treated mice.
Figure 3.7. Treatment with PRN694 has no effect on the progression of cutaneous leishmaniasis. (A) Graph showing size of lesions in vehicle-treated and PRN694-treated mice infected with *Leishmania major* during 6 weeks of infection. The data are presented as mean ± S.E.M of lesion size measurements in total of n=20 mice per group. (B) Graphs show parasite burdens in *L. major* infected vehicle-treated and PRN694-treated mice at weeks 2 and 6 post-infection. The data are presented as mean ± S.E.M of log parasite dilution values in total of n=10 mice per group. Female BALB/c mice (age 8-10 weeks) were subcutaneously injected with 2 million stationary-phase *Leishmania major* promastigotes in the hind footpad. On the same day, infected mice were treated with vehicle or PRN694 administered once daily *via* oral gavage. Lesion sizes were monitored on a weekly basis with a dial-gauge micrometer until 6 weeks post-infection. At weeks 2 and 6 post-infection, infected mice were sacrificed to analyze parasite burden by limiting dilution assay. The data are representative of two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 3.7.

A  Lesion size

![Graph showing lesion size over weeks of infection for Vehicle and PRN694.]

B  Parasite burden

<table>
<thead>
<tr>
<th>Week 2</th>
<th>Week 6</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>Vehicle</td>
</tr>
<tr>
<td>PRN694</td>
<td>PRN694</td>
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</tbody>
</table>

![Bar graphs showing parasite burden for Vehicle and PRN694 at weeks 2 and 6.]

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Figure 3.8. Treatment with PRN694 does not alter T cell proliferation and cytokine production following infection with \textit{L. major}. (A) T cell proliferation, (B) IFN-γ, (C) IL-4 and (D) IL-10 production by \textit{L. major} infected vehicle-treated and PRN694-treated mice at week 2 post-infection. (E) T cell proliferation, (F) IFN-γ, (G) IL-4 and (H) IL-10 production by \textit{L. major} infected vehicle-treated and PRN694-treated mice at week 6 post-infection. The data are presented as mean + S.E.M of sample values from n=10 mice per group. Female BALB/c mice (age 8-10 weeks) were subcutaneously injected with 2 million stationary-phase \textit{Leishmania major} promastigotes in the hind footpad. On the same day, infected mice were treated with vehicle or PRN694 administered once daily \textit{via} oral gavage. At weeks 2 and 6 post-infection, infected mice were sacrificed and draining popliteal lymph nodes were isolated to prepare single cell suspensions. Lymph node cells were stimulated with 20 µg/ml freeze-thaw prepared \textit{L. major} antigen. After 60 hours of stimulation, T cell proliferation was determined by alamarBlue reduction method. After 72 hours of stimulation, culture supernatants were collected to determine the cytokine production by ELISA. The data are representative of two independent experiments. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$. 

\begin{figure}[h] 
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\includegraphics[width=\textwidth]{figure3.8.png} 
\caption{Figure 3.8. Treatment with PRN694 does not alter T cell proliferation and cytokine production following infection with \textit{L. major}. (A) T cell proliferation, (B) IFN-γ, (C) IL-4 and (D) IL-10 production by \textit{L. major} infected vehicle-treated and PRN694-treated mice at week 2 post-infection. (E) T cell proliferation, (F) IFN-γ, (G) IL-4 and (H) IL-10 production by \textit{L. major} infected vehicle-treated and PRN694-treated mice at week 6 post-infection. The data are presented as mean + S.E.M of sample values from n=10 mice per group. Female BALB/c mice (age 8-10 weeks) were subcutaneously injected with 2 million stationary-phase \textit{Leishmania major} promastigotes in the hind footpad. On the same day, infected mice were treated with vehicle or PRN694 administered once daily \textit{via} oral gavage. At weeks 2 and 6 post-infection, infected mice were sacrificed and draining popliteal lymph nodes were isolated to prepare single cell suspensions. Lymph node cells were stimulated with 20 µg/ml freeze-thaw prepared \textit{L. major} antigen. After 60 hours of stimulation, T cell proliferation was determined by alamarBlue reduction method. After 72 hours of stimulation, culture supernatants were collected to determine the cytokine production by ELISA. The data are representative of two independent experiments. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.} 
\end{figure}
CHAPTER 4: SUMMARY AND FUTURE DIRECTIONS

Ibrutinib, a BTK and ITK selective inhibitor, is currently employed for the treatment of certain B cell malignancies (Burger & Buggy, 2013; Dubovsky et al., 2013). In addition to inhibiting the survival of malignant B cells, ibrutinib can also modulate the function of other immune cells such as myeloid cells. From studies which have evaluated the efficacy of ibrutinib in treating diseases where myeloid cells mediate pathogenesis, it is clear that ibrutinib has the potential to alter the migration and function of myeloid cells such as monocytes, macrophages and neutrophils. Hence, further research on how ibrutinib affects the function of other cell types within the myeloid lineage will be useful for expanding the applications of this safe and potent drug. Furthermore, since ibrutinib can inhibit the function of B cells (Burger & Buggy, 2013; Honigberg et al., 2010), it should also be pursued as a drug candidate for diseases where B cells contribute to pathogenesis such as autoimmune diseases and certain infectious diseases. Repositioning currently available FDA-approved drugs for alternate indications such as the treatment of neglected tropical diseases is a cost-effective solution considering that these diseases currently receive limited attention and funding for drug discovery initiatives.

In chapter 2, we demonstrated that ibrutinib enhances the development of dendritic cells (DCs). Furthermore, ibrutinib treatment during development of DCs leads to upregulation of surface molecules which are critical for the activation of adaptive immune responses, including MHC-II and co-stimulatory molecules such as CD80. The
upregulation of DC activation markers and the increase in production of cytokines like IFN-β following LPS stimulation is associated with an enhanced ability of these DCs to activate CD4⁺ T cells. We observed that ibrutinib-treated DCs promote T cell proliferation and cytokine production by CD4⁺ T cells. Ibrutinib also increases the expression of CCR7 in DCs and increases the proportion of lymph node migrating DCs in vivo. These results show that ibrutinib enhances the maturation and LPS-mediated activation of DCs.

Our results could have potential applications in mitigating the symptoms of cancer and the side effects of treatment in certain hematological cancers such as chronic lymphocytic leukemia (CLL). A high percentage of CLL patients display immunodeficiency which is characterized by the lack of immunoglobulin production and defective T cell responses. This immune dysfunction can be observed from early stages of the disease, is perpetuated by the immunosuppressive activity of drugs administered during the treatment phase and also leads to poor immunity against infections commonly observed in CLL patients (Hamblin & Hamblin, 2008; Melchardt, Weiss, Greil, & Egle, 2013). Certain DC based vaccines administered to CLL patients restore T cell function by enhancing IFN-γ production and T cell proliferation with a concomitant decrease in the frequency of regulatory T cells in vivo (Palma et al., 2012). Therefore, DC based therapies have the potential to mitigate immunodeficiency during CLL by improving T cell responses. Our studies provide a rationale for the use of ibrutinib in the development of DC-based vaccines for alleviating immunodeficiency during CLL.

Our results could also be employed for the translation of ibrutinib in the development of DC-based therapies for cancer treatment. DCs play an important role in
the surveillance of the body to detect the presence of cancer cells and as antigen-presenting cells to present tumor-derived antigens to T cells for the initiation of anti-cancer immune responses. Indeed, in various forms of solid cancers such as head and neck, oral, lung cancer etc. the presence of DCs at the primary tumor site is associated with a reduction in metastasis and increase in survival of the patients (Lotze, 1997). However in many forms of cancer, the differentiation and function of DCs is often altered in the vicinity of the tumor, possibly by tumor-derived factors as well as the immunosuppressive tumor microenvironment. For instance, in murine melanoma, a high proportion of tumor-infiltrating DCs could not present tumor antigen nor induce proliferation of T cells (Ataera, Hyde, Price, Stoitzner, & Ronchese, 2011; Stoitzner et al., 2008). In many types of cancer, cancer cells subvert myelopoiesis towards the development of immature myeloid cells such as myeloid derived suppressor cells (MDSCs) (Gabrilovich et al., 2012). Tumor cells in breast cancer actively recruit MDSCs which suppress T cell function. These MDSCs are also critical for the establishment of a premetastatic niche (Gabrilovich et al., 2012; Palucka & Banchereau, 2012; Sceneay et al., 2012). Hence, immunotherapies which reprogram immature DCs and MDSCs into mature myeloid cells, such as mature DCs are likely to enhance tumoricidal T cell responses in cancer patients. In order to achieve this, scientists have employed two main strategies in the development of DC-based therapies. One strategy involves the generation of activated DCs by isolating DC precursors from the patient, stimulating them *ex vivo* and injecting them back into the patient to stimulate T cell responses. Another strategy involves the targeting of immature DCs *in vivo* to reprogram them into mature DCs and activate T cell responses (Palucka & Banchereau, 2012). The success of
these strategies is dependent on the *ex vivo* or *in vivo* generation of mature DCs which have high T cell-stimulatory capacity and show increased expression of lymph node-homing chemokine receptors. However, many DC-based therapies have poor clinical efficacy because DC culture strategies lead to the generation of immature or partially mature DCs (Kalinski et al., 2010). Our results have the potential to alleviate this significant bottleneck in the field of DC immunotherapy. Since ibrutinib enhances the expression of DC activation markers, promotes DC-mediated T cell activation and increases the migration of DCs to the lymph node, it could be incorporated into the cocktail which is used for the *ex vivo* generation of mature DCs for cancer immunotherapy. In this regard, CpG DNA has shown promise as a vaccine adjuvant, particularly for its ability to boost the activity of antigen-presenting cells and promote adaptive immune responses (Bode, Zhao, Steinhagen, Kinjo, & Klinman, 2011; Scheiermann & Klinman, 2014). Moreover, CpG is also being studied as an adjuvant in the development of DC-based therapies for diseases like cancer (Mende & Engleman, 2005). Hence, we studied how ibrutinib treatment modulated the phenotype and function of DCs following activation with CpG-ODN as a stimulus. We observed that the cytokine profile of CpG treated DCs following ibrutinib treatment was similar to LPS/ibrutinib-treated DCs. CpG/ibrutinib-treated DCs showed higher production of IL-10 but lower production of IL-12 compared to CpG only-treated DCs (Figure 4.1A and 4.1B). Unlike ibrutinib-treated DCs stimulated with LPS, CpG/ibrutinib-treated DCs showed significantly higher production of IL-6 compared to CpG only-treated DCs (Figure 4.1C). The levels of TNF-α was similar between both CpG/ibrutinib-treated DCs and CpG only-treated DCs (Figure 4.1D). We also observed that ibrutinib-treated DCs had a higher
proportion of MHC-II⁺ although the expression level of MHC-II in these cells was significantly lower compared to untreated DCs following CpG stimulation. Further, the proportion of CD80⁺ and CD40⁺ DCs as well as the expression of these two molecules was significantly higher in ibrutinib-treated DCs compared to untreated DCs upon CpG stimulation. On the other hand, ibrutinib-treated DCs had a lower percentage of CD86⁺ DCs and showed lower expression of CD86 compared to untreated DCs upon activation with CpG (Figure 4.2A and 4.2B). We also compared the ability of OVA-pulsed CpG/ibrutinib-treated and CpG only-treated DCs to activate the proliferation of CD4⁺ T cells. CD4⁺ T cells cultured with CpG/ibrutinib-treated DCs had a significantly higher percentage of proliferating cells compared to CD4⁺ T cells cultured with CpG only-treated DCs (Figure 4.3). We could not detect the presence of T cell derived cytokines in the culture supernatants, possibly as a result of the overall low levels of T cell proliferation in our study (data not shown). Taken together, these studies show that DCs cultured with ibrutinib during the developmental process display a higher activation status following stimulation with CpG. These results provide a basis for future translational research on the use of ibrutinib in combination with clinically relevant adjuvants like CpG for the development of DC-based therapies for cancer treatment.

In order to study the how ibrutinib affects the differentiation of DCs in the presence of cancer cell products, we cultured DC precursors without or with ibrutinib and supernatants prepared from Polyoma Middle T (PyMT) mammary carcinoma cells to generate untreated, ibrutinib-treated, PyMT only-treated and PyMT/ibrutinib-treated DCs. We then measured cytokine production following stimulation with LPS. PyMT/Ibrutinib-treated DCs showed significantly lower IL-12 production compared to
PyMT only-treated DCs (Figure 4.4A). Further, PyMT/ibrutinib-treated DCs produced higher levels of IL-10 compared to PyMT only-treated DCs (Figure 4.4B). Although these trends of lower IL-12 and higher IL-10 production by PyMT/ibrutinib-treated DCs compared to PyMT only-treated DCs was similar to the cytokine profiles of ibrutinib only-treated and untreated DCs, the differences in cytokine production was more pronounced between PyMT/ibrutinib-treated and PyMT only-treated DCs (Figure 4.4A and 4.4B). We also observed that PyMT/Ibrutinib-treated DCs showed significantly higher TNF-α production compared to PyMT only-treated DCs (Figure 4.4C). There were no differences in IL-6 production between the two groups (Figure 4.4D). These results show that the PyMT supernatants modulate the activation of ibrutinib-treated DCs to dampen IL-12 production and enhance IL-10 and TNF-α production. We observed similar results for LPS-mediated cytokine production when untreated and ibrutinib-treated DCs were cultured with EO771 breast cancer cells (data not shown). To better understand how ibrutinib treatment affects the maturation and activation of DCs in the presence of cancer cells, we will study the effect of ibrutinib in breast cancer in vivo using a murine model of breast cancer. These studies will allow us to determine whether ibrutinib can be employed to enhance the maturation of DCs in vivo and thereby, promote anti-cancer T cell responses. Our future studies will also focus on whether ibrutinib promotes the maturation and activation of human DCs from monocytic precursor cells.

In chapter 3, we demonstrated that treatment with ibrutinib limited the progression of cutaneous leishmaniasis (CL) by *Leishmania major*. Ibrutinib also inhibited the production of Th2-associated cytokines such as IL-4, IL-10 and IL-13 during infection as well as Th2-associated antibody, IgG1. This dampening of Th2
response during *L. major* infection appears to not be dependent on ITK activity, since ITK inhibition using a selective inhibitor, PRN694, did not alter the progression of disease. Further, treatment with PRN694 did not dampen Th2 responses during *L. major* infection. Our study provides a basis for further exploring ibrutinib as an immunotherapeutic drug for the treatment of leishmaniasis.

Standard therapies for leishmaniasis, in various forms of leishmaniasis such cutaneous and visceral leishmaniasis (VL), include drugs that target the parasite such as pentavalent antimonials and amphotericin B. However, the increase in drug resistance, long treatment regimens, toxic side effects and drug costs are major drawbacks of these standard lines of therapy. Immunotherapies that enhance leishmanicidal immune responses in the patient are desirable since they can potentially be used in combination with standard therapies to reduce issues of drug dosage, treatment times and toxic side effects (N. Singh, Kumar, & Singh, 2012; O. P. Singh & Sundar, 2014). Indeed, many such combinatorial approaches using immunotherapies along with chemotherapeutic agents have shown high efficacy in treating both CL and VL. The major strategy in the development of immunotherapies centers on modulating the patients’ immune system to enhance protective Th1 immune responses while inhibiting Th2 responses that promote parasite growth and persistence (O. P. Singh & Sundar, 2014). Since ibrutinib dampens the production of Th2 response, it is a good candidate for combination therapy along with standard drugs. Future studies should study whether ibrutinib can also be employed for the treatment of VL. Further, studies should also evaluate the efficacy of ibrutinib in combination with antimonials such as sodium stibogluconate in treating leishmaniasis. Additionally more analysis into the mechanism by which ibrutinib mediates resistance
against leishmaniasis will be crucial for the translation of this drug for treatment in humans.

Taken together, we hope that our studies on identifying novel indications for ibrutinib, either in the development of dendritic cell-based therapies for cancer treatment or as an immunotherapeutic candidate for treatment of leishmaniasis, will serve as valuable progress towards expanding the scope and usage of this drug.
Figure 4.1. Ibrutinib modulates the production of cytokines of DCs following CpG stimulation. (A) IL-12, (B) IL-10, (C) IL-6 and (D) TNF-α production by untreated and ibrutinib-treated DCs upon CpG stimulation. Bone marrow cells from C57BL/6 mice were cultured in the presence of GM-CSF for 7 days. At Day 1 cultures were treated with PBS or 1µM ibrutinib to generate untreated and ibrutinib-treated DCs respectively. At Day 7, DCs were harvested from untreated and ibrutinib-treated DC cultures and rested for 24 hours prior to further studies. Rested untreated and ibrutinib-treated DC cultures were treated with control (media) or CpG (25 μg/ml). After 24 hours of CpG treatment, cytokine production was determined in the culture supernatants by ELISA. The data are presented as mean ± S.E.M of triplicate sample values from two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 4.2. Ibrutinib differentially regulates the expression of MHC-II and co-stimulatory molecules in CpG-activated DCs. (A) Histograms show the expressions of MHC-II, CD80, CD86 and CD40 in CD11c^+ DCs from CpG/untreated and CpG/ibrutinib-treated DCs. Numbers denote mean ± S.E.M of duplicate percentage values. (B) Mean fluorescence intensities of MHC-II, CD80, CD86 and CD40 on CD11c^+ DCs from untreated and ibrutinib-treated DCs upon CpG stimulation. The data are presented as mean ± S.E.M of duplicate MFI values. Untreated and ibrutinib-treated DCs were treated with control (media) or CpG (25 μg/ml) for 24 hours. After 24 hours, cells were stained with fluorescently labelled antibody for the respective surface molecules and their expressions were determined by flow cytometry. Analyses were conducted by gating on CD11c^+ DCs. The data presented are representative of three independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 4.3. Ibrutinib-treated DCs enhance T cell proliferation following stimulation with CpG. Analysis of T cell proliferation upon co-culture with untreated, ibrutinib only-treated, CpG only-treated or CpG/ibrutinib-treated DCs. Untreated and ibrutinib-treated DCs were pulsed with OVA (10 μg/ml) for 2 hours prior to treatment with CpG (25 μg/ml) for 22 hours. After OVA/CpG stimulation, DCs were cultured in 1:4 ratio with CFSE-stained T cells enriched from spleens of OT-II mice for 6 days. At Day 6, cells from co-culture were stained with anti-CD4 antibody and T cell proliferation was measured by flow cytometry. Analyses were conducted by gating on CD4⁺ population. The data are presented as mean ± S.E.M of duplicate sample values and is representative of two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
Figure 4.4. Culture with PyMT cancer cell supernatants alters the LPS-mediated cytokine responses of ibrutinib-treated and untreated DCs. (A) IL-12, (B) IL-10, (C) TNF-α and (D) IL-6 production by untreated, ibrutinib-treated, PyMT only-treated and PyMT/ibrutinib-treated DCs following LPS stimulation. Bone marrow cells from C57BL/6 mice were cultured in the presence of GM-CSF for 7 days. At Day 1 cultures were treated with PBS or 1μM ibrutinib to generate untreated and ibrutinib-treated DCs respectively. At Day 4, cells were treated with supernatants prepared from resting PyMT breast cancer cells. At Day 7, DCs were harvested and rested for 24 hours prior to further studies. Rested untreated, ibrutinib-treated, PyMT only-treated and PyMT/ibrutinib-treated DCs were treated with control (media) or LPS (1 μg/ml). After 24 hours of LPS treatment, cytokine production was determined in the culture supernatants by ELISA. The data are presented as mean ± S.E.M of triplicate sample values from two independent experiments. *p < 0.05, **p < 0.001, ***p < 0.0001.
REFERENCES


Mukhopadhyay, S., George, A., & Bal, V. (1999). Bruton’s Tyrosine Kinase Deficiency in Macrophages Inhibits Nitric Oxide Generation Leading to Enhancement of IL-12


Ronet, C., Voigt, H., Himmelrich, H., Doucey, M., Torre, Y. H., Tacchini-cottier, F., …


Yang, Y., Shaffer, A. L., Emre, N. C. T., Ceribelli, M., Zhang, M., Wright, G., … Staudt,

