EFFECT OF ADENOSINE DIPHOSPHATE ON DENDRITIC CELL AND T CELL RESPONSES

by

K. Nicole Graves

Submitted in partial fulfillment of the requirements for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
November 2011

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Supervisor: _________________________________

Readers: _________________________________

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_________________________________

Departmental Representative: _________________________________
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This is for you, Cath.
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ABSTRACT

Nucleotides, such as ATP and its derivatives, are released at high concentrations at sites of inflammation and modulate the immune response. When cultured in the presence of ADP or the stable analogue ADPβS, DC surface expression of MHC-II and co-stimulatory molecules, CD40 and CD86 was unchanged. When DCs were pre-treated with ADP or ADPβS, there was no change in their ability to activate naïve CD4+ T cells. However, when CD4+ T cells were activated in the presence of ADP or ADPβS, activation and proliferation were significantly decreased. This correlated with a significant reduction in IL-2 secretion and CD25 surface expression, which may be due to decreased ERK and Akt phosphorylation. CD8+ T cell proliferation was unaffected by the addition of ADP or ADPβS, but secretion of IFN-γ was significantly reduced. By demonstrating that ADP inhibits CD4+ T cell responses, I have identified a potential target of immune modulation by clinical intervention.
<table>
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<td>7-Amino Actinomycin D</td>
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<tr>
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<td>Activator Protein-1</td>
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<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
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<tr>
<td>ADPβS</td>
<td>Adenosine 5’-β-thio Diphosphate</td>
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<tr>
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<td>Adenosine Triphosphate</td>
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<td>Antigen Presenting Cell</td>
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<td>APS</td>
<td>Ammonium Persulfate</td>
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<tr>
<td>BCR</td>
<td>B Cell Receptor</td>
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<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>CFSE</td>
<td>Carboxyfluorescein Diacetate Succinimidyline Ester</td>
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<td>Chemokine (C-C motif) Ligand</td>
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<td>CCR</td>
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<td>Cluster of Differentiation</td>
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<td>Complementary Deoxyribonucleic Acid</td>
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<td>Cmax</td>
<td>Concentration maximum</td>
</tr>
<tr>
<td>CPM</td>
<td>Counts Per Minute</td>
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<tr>
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<td>Cyclic Adenosine Monophosphate</td>
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<tr>
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<td>Cytotoxic T Lymphocyte</td>
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</tr>
<tr>
<td>d</td>
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<tr>
<td>°C</td>
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<td>-----------------------------------------------</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
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<td>Deoxynucleotide Triphosphate</td>
</tr>
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<td>ELISA</td>
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<tr>
<td>ERK</td>
<td>Extracellular Signal Regulated Kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FACS</td>
<td>Fluorescence Activated Cell Sorting</td>
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<td>Foxp3</td>
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<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<td>GAPDH</td>
<td>Glutaraldehyde-3-Phosphate Dehydrogenase</td>
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<tr>
<td>g</td>
<td>Gravity</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte Monocyte-Colony Stimulating Factor</td>
</tr>
<tr>
<td>GRP</td>
<td>Guanyl Nucleotide-Releasing Protein</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>h</td>
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</tr>
<tr>
<td>Ig</td>
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</tr>
<tr>
<td>ITAM</td>
<td>Immunoreceptor Tyrosine based Activation Motifs</td>
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<tr>
<td>IDO</td>
<td>Indoleamine 2, 3-Dioxygenase</td>
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<tr>
<td>ICOS</td>
<td>Inducible Costimulator</td>
</tr>
<tr>
<td>IKKβ</td>
<td>Inhibitor of Nuclear Factor-κB Kinase β</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<td>IL</td>
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<td>Janus Activated Kinase</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>MHC I</td>
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</tr>
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<td>MHC II</td>
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<td>mRNA</td>
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<tr>
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<tr>
<td>µCi</td>
<td>Micro-Curie</td>
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<tr>
<td>µg</td>
<td>Microgram</td>
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<td>Nuclear Factor of Activated T cells</td>
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<td>NF-κB</td>
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<td>NOD</td>
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<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Pattern</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<td>Phosphorylated Akt</td>
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<td>Phosphorylated Extracellular Signal Regulated Kinase</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>pg</td>
<td>picogram</td>
</tr>
<tr>
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<td>PTK</td>
<td>Protein Tyrosine Kinase</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>sec</td>
<td>Second</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>Th</td>
<td>T Helper</td>
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<tr>
<td>Treg</td>
<td>T Regulatory Cell</td>
</tr>
<tr>
<td>T-bet</td>
<td>T-box expressed in T cells</td>
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<td>TEMED</td>
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Firstly, I would like to thank my supervisor, Dr. Robert Liwski, for his unfailing support and guidance through my honours and graduate experience. I will always be grateful for his enthusiasm, which has fostered my love of research. I would also like to extend sincere gratitude to my co-supervisor, Dr. Kenneth West for his insight and advice, and to my committee members, Drs. Brent Johnston and Jean-Francois Légaré, for their direction and assistance.

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CHAPTER 1: INTRODUCTION

1.1 OVERVIEW

Host defenses typically involve two arms of the immune response – innate and adaptive immunity. Innate responses occur rapidly following an immune challenge and result in non-specific targeting of infecting agents. Conversely, adaptive responses take longer to develop and involve specific recognition of the pathogen and the development of long-term immunological memory (Hoebe et al., 2004). Important in bridging innate and adaptive immune responses are dendritic cells (DCs). DCs are professional antigen presenting cells (APCs) that reside in peripheral tissues and act as sentinels of the immune system by engulfing antigen and responding to danger signals, such as pathogen associated molecular patterns (PAMP) or danger associated molecular patterns (DAMP) (Reis e Sousa, 2001, Kono and Rock, 2008). After encountering such signals, DCs undergo maturation and home to draining lymph nodes where they present antigen and activate naïve T cells (Banchereau and Steinman, 1998). Once fully activated, T cells rapidly proliferate and migrate from the lymph node to sites of inflammation (Marelli-Berg et al., 2008). Here, T cells can exert a wide variety of effector functions. T helper (Th) cells are responsible for shaping the adaptive response by releasing cytokines to stimulate and recruit other immune cells, while cytotoxic T lymphocytes (CTL) release mediators to directly and specifically kill pathogen targets (Wong and Pamer, 2003, Liu and Nussenzweig, 2010). Taken together, DCs and T cells play a central role in initiating and executing the adaptive immune response.
1.2 Sentinels of the Immune System

Dendritic cells are derived from myeloid precursors in the bone marrow and are characterized by distinct stellate morphology and surface molecule expression (Banchereau and Steinman, 1998). Following differentiation, immature DCs migrate from the bone marrow to reside in peripheral tissues as sentinel cells (Liu and Nussenzweig, 2010). Some immature DCs migrate to lymph nodes and remain as resident DCs. These DCs respond to danger signals and capture antigen that enters the lymph node by the afferent lymphatics (Sixt et al., 2005). To migrate specifically to sites of inflammation, immature DCs express high levels of chemokine receptors (C-C motif) CCR1, CCR2, and CCR5, which bind a number of pro-inflammatory chemokines (Sallusto et al., 1998). In all tissue sites, immature DCs continuously sample antigen by various endocytic mechanisms, including phagocytosis, receptor-mediated endocytosis, and macropinocytosis (Sallusto et al., 1995).

Immature DCs must be able to respond rapidly to cell distress, including infection and trauma. To detect such danger signals, immature DCs express high levels of pattern recognition receptors (PRRs), which recognize PAMPs, and various receptors that can bind DAMPs (Kono and Rock, 2008, Trinchieri and Sher, 2007). The term PAMP describes a wide variety of microbial products that are highly conserved and bind specific PRRs to elicit a response, such as lipopolysaccharide (LPS) stimulation of toll like receptor (TLR)4 to activate immature DCs (Agrawal et al., 2003). Conversely, DAMPs, which also promote specific responses, are cellular antigens that are only exposed to the extracellular environment following necrosis or shear stress (Bianchi, 2007). DAMPs can include chromatin, nucleosomes, uric acid, and important for this study, adenosine...
triphosphate (ATP) and derivative nucleotides (Fredholm, 2007, Kono and Rock, 2008). Activation of DCs by PAMP and DAMP recognition initiates the maturation process and is central to bridging innate and adaptive immunity.

1.2.1 DENDRITIC CELL MATURATION

Mature DCs are professional APCs capable of initiating responses from naïve T cells by providing three distinct signals (Hugues, 2010). Following innate activation, DCs mature through a process by which antigen capture is decreased, and antigen processing and presentation is increased (Sallusto et al., 1995). Antigens engulfed in the periphery are processed via the endosomal/lysosomal pathway, with peptides being generated by proteolytic enzyme activity (Ferrari et al., 1997). Within endosomal compartments, major histocompatibility complex-II (MHC-II) is loaded with peptides and the complex is trafficked to the DC surface (Ferrari et al., 1997, Chow et al., 2002). Alternatively, extracellular antigen can be captured, processed and cross-presented in the context of MHC-I (Heath et al., 2004). Interaction of MHC-II or MHC-I with the T cell receptor (TCR) on CD4+ or CD8+ T cells, respectively, constitutes signal 1 for T cell activation (Hugues, 2010, Mempel et al., 2004).

Activation of immature DCs by PAMPs and DAMPs also causes up-regulation of co-stimulatory molecules cluster of differentiation (CD)40, CD80, and CD86 (Fujii et al., 2004) and actin-cytoskeleton rearrangement (Al-Alwan et al., 2003) for subsequent activation of naïve T cells. Interactions of co-stimulatory molecules on DCs and T cells are regarded as ‘signal 2’ and are necessary for full activation of naïve T cells (Hugues, 2010, Mempel et al., 2004).
2010, Liwski et al., 2006). Binding of CD40 on DCs to CD40 ligand (CD40L) on T cells promotes DC survival and continued DC activation (Caux et al., 1994), whereas prolonged interaction of CD80/CD86 on DCs with T cell CD28 is required for naïve T cells to become activated and begin proliferating (Liwski et al., 2006).

Mature DCs also have increased pro-inflammatory cytokine production, which aids in T cell activation (signal 3), including tumor necrosis factor (TNF) and IL-6 (Geginat et al., 2003). Furthermore, cytokine secretion by DCs modulates adaptive immune cell responses, and in particular, causes polarization of T cell responses (Banchereau and Steinman, 1998). Depending on the cytokine milieu at the time of activation, naïve T cells can adopt different Th phenotypes (Zhu et al., 2010), which will be discussed in detail below.

1.2.2 DENDRITIC CELL MIGRATION

During the maturation process, DCs migrate from sites of inflammation to T cell areas in draining lymph nodes or the spleen. Mediating this migration is a switch in chemokine receptor expression. Activation of DCs causes down-regulation of immature DC chemokine receptors (CCR1, CCR2, and CCR5) and up-regulation of CCR7 (Sallusto et al., 1998). CCR7 is responsive to the chemokine ligands (C-C motif) CCL19 and CCL21, which are constitutively expressed in lymphatic tissue (Sozzani et al., 1998, Yoshida et al., 1998). Interactions between CCR7 and CCL21 mediate DC entry into draining lymph nodes (Tal et al., 2011). Once in T cell areas, DCs are able to present antigen in the context of MHC and activate naïve T cells by co-stimulatory molecule interactions.
1.3 T CELL ACTIVATION

Naïve T cells develop in the thymus from lymphoid progenitor cells and are generally classified by surface expression of either CD4 or CD8 (Takahama, 2006). Following thymic development, naïve CD4+ and CD8+ T cells enter the circulation through interaction with selectin and integrin adhesion molecules, which mediate leukocyte rolling, tethering and extravasation through the endothelial monolayer (Marelli-Berg et al., 2008). Naïve T cells are then directed toward the lymphatics and secondary lymphoid organs by CCL19 and CCL21 interaction with CCR7 (Takahama, 2006, Ueno et al., 2002). Within secondary lymphoid tissue, such as lymph nodes or the spleen, T cells remain in an inactive state until they communicate with an APC. DCs are the most potent APC and efficiently induce an effector T cell response. Although other APCs, such as macrophages and B cells, are capable of activating T cells, the T cell response elicited is not as strong (Reichardt et al., 2010). As such, I will focus on DC-mediated T cell activation.

1.3.1 T CELL RECEPTOR SIGNALING

DCs present antigen to naïve T cells in the context of MHC, allowing recognition of the antigenic peptide by the TCR (Smith-Garvin et al., 2009). Ligation of the TCR activates protein tyrosine kinases (PTK; lck and fyn), which phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) on the closely associated CD3 complex (Samelson et al., 1986, Qian et al., 1993). Phosphorylation of CD3 allows recruitment of zeta-chain-associated protein kinase-70 (ZAP-70), which can activate a
number of signaling pathways. Importantly, ZAP-70 phosphorylates the adaptor protein linker for the activation of T cells (LAT), causing downstream activation of phospholipase C-γ1 (PLC-γ1), which hydrolyses phosphatidylinositol-4,5-bisphosphate (PIP2) to produce second messengers, inositol trisphosphate (IP3) and diacylglycerol (DAG) (Smith-Garvin et al., 2009, Reynolds et al., 2002)).

Production of DAG causes recruitment of protein kinase C theta (PKCθ), which can contribute to two different signaling events. PKCθ can activate nuclear factor-κ-B (NF-κB) by phosphorylating inhibitor of NF-κB kinase (IKK), causing phosphorylation of inhibitor of κB (IκB), allowing release of NF-κB and its translocation to the nucleus (Coudronniere et al., 2000, Lin et al., 2000). PKCθ can also phosphorylate Ras guanyl nucleotide-releasing protein (RasGRP), causing the release of activated Ras (RasGTP) (Finco et al., 1998). RasGTP stimulates downstream mitogen-associated protein kinases (MAPKs) to culminate in the activation of extracellular signal-regulated kinases (ERK)1 and ERK2 by phosphorylation (Smith-Garvin et al., 2009). Activated ERK1 and ERK2 have an important role in initiating proliferative responses in activated T cells due to promotion of cell cycle progression (Meloche and Pouyssegur, 2007). Furthermore, activated ERK1 and ERK2 mediate activation of Fos, a transcription factor that complexes with Jun to form activator protein-1 (AP-1) (Rauscher et al., 1988), which induces production of T cell growth factor interleukin (IL)-2 (Whitehurst and Geppert, 1996). Importantly, activated ERK1/2 also promotes survival of activated T cells by inhibiting the pro-apoptotic protein Bim (O'Reilly et al., 2009).

Production of the second messenger IP3 causes release of calcium (Ca2+) from intracellular stores (Feske et al., 2003). Increased intracellular Ca2+ activates calmodulin,
leading to calcineurin-mediated activation of nuclear factor of activated T cells (NFAT) (Feske et al., 2003, Oh-hora, 2009), a transcription factor involved in IL-2 production and T helper cell differentiation (reviewed by (Oh-hora, 2009, Macian, 2005). Furthermore, intracellular Ca\(^{2+}\) can also cause activation of calpain, which may contribute to actin rearrangements during formation of the immunological synapse (Selliah et al., 1996, Burkhardt et al., 2008).

1.3.2 **CO-STIMULATION**

While interactions between MHC and the TCR stimulate important T cell transcription factors including NF-κB, AP-1, and NFAT, the interactions are not sufficient to fully activate naïve T cells. In the absence of co-stimulation, T cells enter a state of anergy, in which antigen-specific T cells become unresponsive and exhibit impaired proliferation and cytokine production (Lucas et al., 1995, Wells, 2009). Co-stimulation, which is required to enhance TCR-mediated T cell activation, can occur through ligation of various molecules, including CD28, CD40L, inducible co-stimulator (ICOS), and OX40 (Smith-Garvin et al., 2009).

The best characterized co-stimulatory molecule is CD28, which binds CD80 and CD86 on APCs (Acuto and Michel, 2003). Prolonged co-stimulation of T cells by CD28 interaction with CD80/CD86 is essential as it enhances T cell proliferative and effector capabilities (Liwski et al., 2006). CD28 augments responses initiated by MHC-TCR interaction to prevent T cells from becoming anergic (Smith-Garvin et al., 2009). CD28 co-stimulation is required for full transcriptional activity of AP-1 (Rincon and Flavell,
1994), and is also necessary for full stimulation of NF-κB and NFAT (Smith-Garvin et al., 2009). Specifically, CD28 ligation causes association of phosphoinositide-3-kinase (PI3K), which phosphorylates PIP2 to form phosphatidylinositol-3, 4, 5-trisphosphate (PIP3) (Pages et al., 1994). Generation of PIP3 causes recruitment of 3-phosphoinositide-dependent protein kinase-1 (PDK-1) and subsequent phosphorylation of the serine/threonine kinase, Akt, which has a number of downstream activities that enhance T cell activation (Kane and Weiss, 2003). Activated Akt can induce translocation of NF-κB to the nucleus, resulting in altered gene expression patterns (Kane et al., 2002).

Specifically, Akt-induced NF-κB activity causes up-regulation of the pro-survival protein BclXL (Boise et al., 1995, Burr et al., 2001) and increased transcription of pro-inflammatory cytokine genes (Cheng et al., 2011). Importantly, Akt enhances NFAT activity by preventing its nuclear export by glycogen-synthase kinase-3 (GSK-3) (Beals et al., 1997) and also synergizes with PKCθ to increase production of IL-2 (Lin et al., 2000, Kane et al., 2001). Co-stimulation through CD28 and OX-40 also increases IL-2 and IFN-γ mRNA stability, further promoting enhanced production of these pro-inflammatory cytokines (Mestas et al., 2005, Miller et al., 2009). Representation of TCR- and CD28-mediated signaling pathways is shown in Figure 1.

1.3.3 **AUTOCRINE INTERLEUKIN-2 SIGNALING**

Importantly, co-stimulation of T cells results in increased production of IL-2, which has long been recognized as an essential growth factor for T cells (Smith, 1980). Following activation through MHC-TCR interactions and co-stimulation, T cells rapidly
increase surface expression of the IL-2 receptor, a trimeric protein consisting of an α-, β- and common γ-chain (Malek and Bayer, 2004). Expression of the β- and γ-chains is not limited to T cells, and are components of other cytokine receptors (He et al., 1997). However, the α-subunit (CD25) is highly expressed specifically on activated and regulatory T cells (Treg) to allow high-affinity binding of IL-2 (Malek and Bayer, 2004).

Enhanced production of IL-2 induces robust proliferative responses of T cells in vitro by signaling through the IL-2 receptor in an autocrine manner. To initiate this response, IL-2 binds CD25, causing association of the β- and γ-subunits. Assembly of the IL-2 receptor induces cross-phosphorylation of Janus activated kinase (JAK)1 and JAK3 and downstream activation of MAPK and Akt signaling leading to cell cycle induction (Malek and Bayer, 2004). Furthermore, JAK1/JAK3 phosphorylation activates signal transducer and activator of transcription (STAT)5, which enhances T cell proliferation and pro-inflammatory gene expression (Moriggl et al., 1999, Malek and Castro, 2010). Interestingly, production of IL-2 is not essential during in vivo T cell responses, but maintains an important role in promoting expansion of effector cells along with additional cytokines including IL-4, IL-6, IL-12, and TNF (Geginat et al., 2003, Malek et al., 2001).

1.4 CD4+ T Cell Subsets

The cytokine milieu during T cell activation largely shapes the adaptive immune response. Depending on the inflammatory stimulus, innate effector cells (including DCs) secrete cytokines to drive differentiation of CD4+ T cells into distinct subsets (de Jong et
al., 2005), namely T helper (Th)1, Th2, Th17 and Treg populations (Zhu et al., 2010). These subsets have distinct immunological roles, which will be described below.

1.4.1 **THE TH1/TH2 PARADIGM**

In describing T cell effector responses, two subsets were initially identified – Th1 and Th2 cells. Distinction between these lineages was based primarily on selective cytokine production, with Th1 cells being characterized by interferon (IFN)-\(\gamma\) secretion, and Th2 cells by IL-4 secretion (Mosmann et al., 1986). We now know that commitment to either subset is promoted by the cytokine milieu that is present during T cell activation. Specifically, secretion of IFN-\(\gamma\) and IL-12 by DCs induces a Th1 response (Manetti et al., 1993), which is characterized by the secretion of IFN-\(\gamma\), lymphotoxin, and IL-2 (Zhu et al., 2010, Manetti et al., 1993). Alternatively, Th2 responses are induced by DC secretion of IL-6, and results in IL-4, IL-5 and IL-13 production by T cells (Kopf et al., 1993, Zygmunt and Veldhoen, 2011, Diehl et al., 2002). Both Th1 and Th2 subsets have been noted to produce TNF (Zhu et al., 2010).

The differential cytokine secretion by Th1 and Th2 cells can be attributed to the expression of subset-specific transcription factors. Commitment of cells to the Th1 lineage is mediated by increased expression of the transcription factor T-bet (T-box expressed in T cells), resulting in enhanced gene transcription of IFN-\(\gamma\) (Szabo et al., 2000). Importantly, Th1 responses are involved in clearance of intracellular bacterial and viral infections, and have an important role in anti-cancer immunity (Kidd, 2003). Polarization of Th2 responses involves increased expression of the transcription factor
Gata3. Interestingly, Gata3 is down-regulated in Th1 cells, indicating opposing functions of these subsets (Zhang et al., 1997, Zheng and Flavell, 1997). Furthermore, Th2 responses are involved in allergy and immunity against extracellular parasites (Kidd, 2003).

While cytokines play a major role in determining T cell fate following activation, other factors can alter Th1 versus Th2 polarization. Differentiation can depend on antigen affinity, with high affinity binding to the TCR promoting a Th1 response preferentially (Zygmunt and Veldhoen, 2011, Chaturvedi et al., 1996). Additionally, increased antigen load and sustained ERK1/2 activation following TCR engagement may inhibit Gata3 to decrease the Th2 response (Jorritsma et al., 2003).

1.4.2 TH17 CELLS

The most recently described CD4+ T cell subset are the Th17 cells, which develop in response to DC secretion of transforming growth factor (TGF)-β and IL-6 (Veldhoen et al., 2006). Th17 cells are characterized by expression of the orphan nuclear receptor RORγt, and to a lesser extent, RORα (Ivanov et al., 2006, Dong, 2009). Expression of these transcription factors induces secretion of IL-21, which further promotes Th17 differentiation, and IL-17 and IL-22, which are important in various immune states (Dong, 2009). Notably, IL-17 has been implicated in autoimmunity, including animal models of multiple sclerosis and arthritis (reviewed by (Kimura and Kishimoto, 2011). Furthermore, Th17 cells are integral in shaping adaptive responses in clearance of extracellular bacteria (Dong, 2009).
1.4.3 REGULATORY T CELLS

Regulatory T cells (Tregs) have an important physiological role, as highlighted by their involvement in peripheral tolerance, autoimmune regulation and control of chronic inflammation (Vignali et al., 2008). Naturally occurring Tregs (nTregs) mature in the thymus, and are characterized by constitutive transcriptional activity of forkhead box protein-3 (Foxp3) (Chatila, 2005). DC stimulation by the cytokines IL-10 and TGF-β induces Tregs in the periphery, causing increased activity of Foxp3 (reviewed by Zygmunt and Veldhoen, 2011). Foxp3 is required for maintenance of Treg suppressor activity (Fontenot et al., 2003).

In mice, natural and induced Tregs tend to have similar characteristics in vitro and in vivo. Both subsets are characterized by the production of cytokines TGF-β, IL-10 and IL-35, which contribute to negative regulation of effector T cell responses (Vignali et al., 2008, Collison et al., 2007). Additionally, Tregs display high surface expression of the high affinity IL-2 receptor, CD25. Expression of CD25 by Tregs is thought to contribute to suppressor activity, as it depletes extracellular IL-2 and induces cytokine-deprivation-mediated apoptosis of effector T cells (de la Rosa et al., 2004). Furthermore, immunosuppressive cytokines produced by Tregs are able to directly diminish DC-mediated T cell activation by inducing DC production of the inhibitory molecule indoleamine 2, 3-dioxygenase (IDO) (Zhu et al., 2010).
1.5 Cytotoxic T Lymphocyte Responses

Cytotoxic T lymphocytes (CTLs) are CD8+ T cells that play a major role in immune identification and clearance of pathogen-infected or cancerous host cells (Wong and Pamer, 2003). All nucleated host cells process endogenous peptide antigens and present them on the cell surface in the context of MHC-I (Pamer and Cresswell, 1998). Cells infected with virus or intracellular bacteria consequently present foreign antigen on their cell surface, making themselves targets for CTL activity. Additionally, CTLs are able to recognize and target cancer cells which present altered peptides in MHC-I (Wong and Pamer, 2003). DCs present exogenous antigen to CTL by cross-presentation in MHC-I (Albert et al., 1998).

Similar to CD4+ T cell activation, CD8+ CTLs require three signals: interaction of MHC and the TCR, co-stimulation, and cytokine help (Mescher et al., 2006, Shrikant et al., 2010). Also, CD4+ T cells ‘help’ the induction of CD8+ CTL responses by licensing DCs through CD40 and CD40L interactions (Ridge et al., 1998). While ligation of CD28 induces cell division and promotes survival in CTLs (Sepulveda et al., 1999), IL-12 in the cytokine milieu is required for clonal expansion of CTLs (Mescher et al., 2006, Curtsinger et al., 1999). Interestingly, IL-2 is not required for the induction of CTL proliferation, but it contributes to sustained T cell proliferation (D'Souza and Lefrancois, 2003). Activated CD8+ T cells produce IFN-γ (Sad et al., 1995), a cytokine that promotes CD8+ T cell proliferation, as well as CTL effector responses (Sercan et al., 2006).

Directed killing by CTLs is mediated by the generation of lytic molecules, which are secreted in granules. Specifically, CTLs release perforin to induce pore formation in the target cell (Masson and Tschopp, 1985), and serine proteases called granzymes which
promote target cell apoptosis by activation of caspases (Bossi and Griffiths, 2005). CTLs also up-regulate surface expression of Fas ligand, which binds target cell Fas, to initiate apoptosis (Fan and Zhang, 2005).

1.6 B CELLS

Immature B cells develop in the bone marrow from lymphoid precursors and predominantly reside in secondary lymphoid tissues (Cerutti et al., 2011). B cells become activated following detection of inflammatory stimuli by PRRs, such as the TLR family, and specific antigen recognition by the B cell receptor (BCR) (Pasare and Medzhitov, 2005, Nimmerjahn and Ravetch, 2010). Activation results in a wide array of B cell effector functions, including antibody production, antigen presentation, and development of immunologic memory (reviewed by Cerutti et al, 2011). B cells have an integral role in both innate and adaptive immunity. Antibody production can promote activation of innate cells through the Fc-\(\gamma\) receptor, and can mediate specific effector functions, such as antibody-dependent cell cytotoxicity and opsonization of pathogens for phagocytosis (Nimmerjahn and Ravetch, 2010).

B cells are important APCs that can expand DC-driven T cell responses, especially during secondary immune responses. Like DCs, B cells present antigen in the context of MHC, and fully activate T cells by CD86-CD28 co-stimulation (Rivera et al., 2001). B cells also secrete cytokines, which regulate and shape the adaptive T cell response (Lund, 2008). Importantly, B cells proliferate robustly in response to inflammatory stimuli, such as LPS, thereby propagating the immune response (Cerutti et al., 2011, Minguet et al., 2008).
1.7 **EXTRACELLULAR NUCLEOTIDES AT SITES OF INFLAMMATION**

Nucleotides are small molecules that are generated following phosphorylation of the nucleosides cytidine, uridine, guanosine, thymidine, and adenosine. Nucleotides are constitutively present in the intracellular environment and have a central role in many normal cell processes, including nucleic acid synthesis, and with respect to ATP, intracellular energy transport (reviewed by (Deaglio and Robson, 2011). Since nucleotides are essential for cellular growth and metabolism, to learn that they could have an intercellular role was unexpected. The first evidence of this phenomenon was provided by Burnstock et al. (1970) who determined that ATP was released from non-adrenergic nerves in the gut smooth muscle and was responsible for inhibitory nerve control. Since the time of these landmark studies, the role of ATP and other nucleotides in intercellular signaling has become more clearly identified in cardiac function, glycogen metabolism, homeostasis of the central nervous system and, important for the current study, regulation of immune responses (reviewed by Burnstock and Knight, 2004, Franke and Illes, 2006).

Physiologically, ATP is largely intracellular, with basal extracellular concentrations ranging between 10 and 100 nM, compared with an intracellular concentration of 5-10 mM (Adinolfi et al., 2005, Di Virgilio et al., 2009). Release of ATP to the extracellular environment usually results from cell or tissue damage and acts as a danger signal to alert the innate immune system through ligation of the P2 family of purinergic receptors (Di Virgilio et al., 2001, Bours et al., 2006). Notably, endothelial cells, which line blood vessels, can release ATP following injury due to shear stress or stimulation by PAMPs such as LPS (Bodin and Burnstock, 1998). Also, ATP can be released from necrotic cells,
such as those found within the hypoxic core of a tumor (Pellegatti et al., 2008, Aymeric et al., 2010) to initiate an immune response.

1.7.1 ATP AND THE IMMUNE RESPONSE

Since ATP is an endogenous danger signal, its release at sites of inflammation can cause rapid initiation of the immune response (la Sala et al., 2003). Specifically, release of ATP from cells at sites of injury or tumor can activate the NLRP3 (part of the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family) inflammasome and aid in subsequent induction of IL-1β production (Iyer et al., 2009). IL-1β is important for the activation of various innate neutrophil and macrophage responses, and is critical for CD8+ T cell priming within the tumor microenvironment (Iyer et al., 2009, Ghiringhelli et al., 2009). Interestingly, since ATP can be released from dying cancer cells (Aymeric et al., 2010), establishment of a metastatic site within the lymph node could increase the concentration of extracellular ATP, thereby indicating that ATP could have direct activity on T cells during activation. Release of ATP can modulate innate inflammatory cell recruitment directly by increasing adhesion molecule expression on macrophages (Goepfert et al., 2001, Kronlage et al., 2010) or increasing chemotaxis of immature DCs by induction of cytoskeletal restructuring (Idzko et al., 2002, la Sala et al., 2003). ATP can also indirectly affect immune cell recruitment by reducing secretion of IL-12 and TNF by DCs, promoting a non-Type 1 adaptive response (la Sala et al., 2001, Wilkin et al., 2002, Marteau et al., 2004, Schnurr et al., 2005).

Collectively, the observed effects of ATP may be explained as being part of immune response regulation. Following a traumatic event, ATP is released at high
concentrations from damaged cells, lending to a potent type 1 adaptive response (Bours et al., 2006). As the concentration of ATP diminishes by sequential degradation of ATP to adenosine diphosphate (ADP), adenosine monophosphate (AMP) and adenosine, different responses may be induced by specific P1 or P2 receptor ligation, which may include dampening of ATP-stimulated immunity (reviewed by Deaglio and Robson, 2011, la Sala et al., 2003).

1.7.2 ECTONUCLEOTIDASES

Following stimulation of the immune response, ATP is rapidly degraded in the extracellular environment by the ectonucleotidase class of enzymes. Specifically, two ectonucleotidases, CD39 and CD73, are responsible for ATP break down and regulation of purinergic signaling (Deaglio and Robson, 2011). CD39 is a nucleoside triphosphate diphosphohydrolase that is highly expressed on endothelial cells, monocytes, DCs, and Tregs, and is responsible for the conversion of ATP to ADP and AMP, and ADP to AMP (Koziak et al., 1999, Mizumoto et al., 2002). Conversely, CD73 is an ecto-5’-nucleotidase and completes ATP degradation by generating adenosine from AMP on various cell types (Resta et al., 1998).

Within the T cell subsets, expression of both CD39 and CD73 are restricted to Tregs, allowing for phenotypic distinction between this subset and other effector T cells, along with CD25 and Foxp3 expression (Kobie et al., 2006, Deaglio et al., 2007). By ectonucleotidase activity, Tregs are able to generate high concentrations of adenosine from extracellular nucleotides, contributing to their immunosuppressive activity (Deaglio et al., 2007, Mandapathil et al., 2010).
1.8 **ADENOSINE IS IMMUNOSUPPRESSIVE**

Accumulation of adenosine in the extracellular environment can serve to dampen or suppress the inflammatory response following ATP-mediated stimulation. Adenosine acts through one of four transmembrane G-protein coupled receptors, named A₁, A₂a, A₂b, and A₃, which are widely expressed on immune effector cells (reviewed by (Hasko et al., 2008). However, most of the described immunosuppressive effects of adenosine are initiated through ligation of A₂a, and to a lesser extent, A₂b (Hasko et al., 2008, Linden, 2011). A₂a receptors are coupled to the Go₅-protein, resulting in activation of adenylate cyclase and subsequent cyclic AMP (cAMP) generation (Ralevic and Burnstock, 1998). Furthermore, A₂a ligation has been shown to increase activity of MAPK pathways, and phosphorylation of ERK1/2 in a number of cell types (Schulte and Fredholm, 2003).

1.8.1 **Effect on DC**

When in an immature state, DCs express low levels of the A₂a receptor. Following maturation, DCs upregulate A₂a, allowing them to become sensitive to adenosine in the extracellular environment (Schnurr et al., 2004, Panther et al., 2001). Exposure to adenosine during maturation causes a significant decrease in release of the pro-inflammatory cytokines IL-12 and TNF, while increasing IL-10 and TGF-β production (Panther et al., 2003, Novitskiy et al., 2008). Adenosine treatment of DCs also causes decreased secretion of various chemokines, which prevents DC-mediated recruitment of other innate effector cells to sites of inflammation (Kumar and Sharma, 2009). Furthermore, adenosine-treated DCs exhibit increased transcription of the
immunosuppressive molecule IDO, which promotes Treg induction (Novitskiy et al., 2008). Although adenosine does not inhibit the ability of DCs to present antigen, differentiation of T cells activated by adenosine-treated DCs appears to be skewed to a non-Th1 phenotype (Panther et al., 2003).

1.8.2 EFFECT ON T CELL RESPONSES

In addition to the effects on DC responses, adenosine is directly suppressive to T cell-mediated immunity. Adenosine profoundly inhibits IFN-γ, IL-12 and IL-4 production, thereby diminishing both Th1 and Th2 responses (Sevigny et al., 2007, Csoka et al., 2008, Romio et al., 2011), which Romio et al. (2011) suggest to be due to inhibited NF-κB activity. Furthermore, ligation of the A2a receptor by adenosine decreases T cell proliferative capacity due to a decrease in STAT5 phosphorylation, leading to inhibited IL-2 receptor activity (Zhang et al., 2004). Specifically, T cells activated in the presence of adenosine produce less IL-2 and have attenuated surface expression of CD25, preventing IL-2 mediated clonal expansion (Erdmann et al., 2005). Additionally, adenosine decreases cytotoxic activity by CTLs, by decreasing exocytosis of cytolytic granules, and reducing surface expression of Fas ligand (Koshiba et al., 1997, Hoskin et al., 2008).

The immunosuppressive properties of adenosine can be beneficial in many respects, such as in autoimmune states or following transplantation (Hoskin et al., 2008). However, it must be noted that since adenosine is generated due to metabolic stress and conditions of hypoxia in the tumor microenvironment, adenosine-mediated suppression of the immune response can be quite detrimental to immune surveillance and tumor rejection by
1.9 PURINERIC RECEPTORS

As mentioned briefly above, interaction between the P2 family of purinergic receptors and ATP or the nucleotide derivates, ADP and AMP, can lead to the generation of different physiological responses (Deaglio and Robson, 2011). This family of receptors is divided into two sub-families, the P2X and P2Y receptors. The currently described P2X receptors (P2X1-7) are ligand gated ion channels that interact exclusively with ATP (Burnstock and Knight, 2004), but are beyond the scope of this study. Alternatively, the P2Y receptors (P2Y1, 2, 4, 6, 11-14) are coupled to heterotrimeric G-proteins and have specific binding affinities for ATP, ADP and other uridine nucleotides (Burnstock and Knight, 2004, Erlinge, 2011). The P2Y receptors, P2Y1, P2Y12, and P2Y13 (Webb et al., 1993, Foster et al., 2001, Zhang et al., 2002), bind the ATP derivative, ADP with high affinity. These three P2Y receptors are present in a wide variety of tissues, but are predominately expressed in the central nervous system, and on platelets, and various immune cells inducing DCs and lymphocytes (Burnstock and Knight, 2004, Erlinge, 2011).

As mentioned, the G-coupled proteins mediate signaling by P2Y receptors; P2Y1 is coupled to Goq, and P2Y12/13 are coupled to Goi (Foster et al., 2001, Zhang et al., 2002, Leon et al., 1997). P2Y1 responses involve stimulation of PLCβ and generation of DAG and IP3 from PIP2. Formation of DAG activates downstream MAPK pathways, while IP3 initiates mobilization of intracellular Ca2+ (Ralevic and Burnstock, 1998, Shen and DiCorleto, 2008). Although not described for P2Y1 signaling, Goq inhibits activation of
PI3K, which leads to subsequent inhibition of Akt (Harris et al., 2006). Conversely, P2Y_{12} and P2Y_{13} responses, mediated by G_{\alpha_i}, cause inhibition of adenylate cyclase, and consequently inhibit formation of cAMP, which can negatively affect downstream signaling by PI3K (reviewed by (Burnstock and Knight, 2004, von-Kgelgen and Harden, 2011). While the role of P2Y_{12} and P2Y_{13} in ERK1/2 phosphorylation is remains to be fully elucidated, G_{\alpha_i} signaling is associated with decreased ERK1/2 activity in other model systems (Goldsmith and Dhanasekaran, 2007).

### 1.10 Role of ADP

The role of ADP in inflammatory responses has yet to be fully elucidated. Although ADP is a short-lived mediator due to ectonucleotidase activity (Deaglio and Robson, 2011), it binds with high affinity to the P2Y_{12} and P2Y_{13} receptors (and to a lesser extent, P2Y_{1}) (Erlinge, 2011). Importantly, ligation of ADP by P2Y_{1} and P2Y_{12} causes activation of platelets, which is a rapid and essential physiological process (Jin et al., 1998, Dorsam and Kunapuli, 2004). Since ADP would be present at sites of inflammation following tissue injury due to ATP break down, it is possible that ADP has effects on neighboring cells.

Within physiological systems, ADP appears to have pro-inflammatory activity. As mentioned, ADP potently induces platelet aggregation in thrombosis (Jagroop et al., 2003), which can lead to the activation of the complement cascade (Del Conde et al., 2005). ADP also induces activation, proliferation and migration of endothelial cells during wound healing (Shen and DiCorleto, 2008, Lyubchenko et al., 2011). Furthermore, use of the P2Y_{12} antagonist, clopidogrel, has been shown to reduce
inflammation in a number of models, including diabetes (Angiolillo, 2006), transplant arteriosclerosis (Abele, 2009) and LPS-induced sepsis (Hagiwara et al., 2011).

A few groups have investigated the role of ADP in DC-mediated immunity. Treatment of DCs with ADP initiates release of Ca\(^{2+}\) from intracellular stores, which is required for activation of the ERK1/2 pathway in human DC (Idzko et al., 2002, Marteau et al., 2004). ADP stimulates receptor-mediated endocytosis, and one group suggested that DCs loaded with antigen in the presence of ADP have enhanced antigen presentation to naïve T cells due to increased antigen uptake (Ben Addi et al., 2010). However, DC production of IL-12 and IL-10 is significantly inhibited (Marteau et al., 2004, Ben Addi et al., 2010), which may affect the resulting T cell response. To date, the effect of ADP on T or B cells has not been directly investigated.

1.11 STUDY RATIONALE AND OBJECTIVES

Recognition of foreign or aberrant bodies by the host is essential for their rapid clearance by the immune system. Activation of immune cells occurs by interaction with various PAMPS and DAMPs, including ATP, leading to initiation of the adaptive response. ATP is an endogenous ‘danger signal’, and its release at sites of tissue injury initiates strong immunogenic responses (Di Virgilio et al., 2001, Bours et al., 2006). Ectonucleotidases degrade ATP sequentially to generate adenosine, a potent immunosuppressive molecule, which can dampen immune activation (Linden, 2011).

ADP, generated during ATP break down, activates distinct purinergic receptors, P2Y\(_{1}\), P2Y\(_{12}\), and P2Y\(_{13}\), which are expressed on a wide host of tissues, including
lymphoid organs. Despite the presence of cognate receptors, few studies have fully investigated the effect of ADP on immune cells. Specifically, studies involving ADP and DC-mediated T cell activation are lacking. Thus, the objective of this study was to determine the role of ADP in control of DC and T cell responses. Given that ADP has pro-inflammatory activities, including potent activation of platelets, I hypothesized that ADP would enhance DC-mediated T cell responses. Through \textit{in vitro} investigation, I examined the effect of ADP on DC maturation by expression of MHC-II and co-stimulatory molecules. DC-driven CD4$^+$ T cell activation and proliferation in the presence of ADP were investigated, with exploration into the effect of ADP on CD8$^+$ T cell responses. Finally, a pilot study into B cell responses in the presence of ADP was performed.
Figure 1. Signal transduction following T cell activation by signal 1 and signal 2. TCR engagement by APC MHC (signal 1) and CD28 ligation by CD80/CD86 (signal 2) lead to a sequence of downstream signaling events, which culminate in activation of the transcription factors AP-1, NFκB, and NFAT. These transcription factors are involved in promoting T cell survival, growth, and proliferation following activation.
CHAPTER 2: MATERIALS & METHODS

2.1 ANIMALS

Female 6-8 week old C57BL/6 and OT-II transgenic mice were purchased from Charles River Laboratories (St. Constant, QC) and housed in the Carleton Animal Care Facility at Dalhousie University (Halifax, NS). Food and water were provided ad libitum. All animal work was done under the approval of Dalhousie University Committee on Laboratory Animals (UCLA) office of animal research oversight.

2.2 SOLUTIONS AND REAGENTS

Complete media was prepared by supplementing RPMI 1640 medium (Fisher Scientific, Ottawa, ON) with 10% fetal bovine serum (FBS; Invitrogen, Burlington, ON), 200 U/mL penicillin, 200 μg/mL streptomycin (Invitrogen), 10 mM Hepes buffer (Invitrogen), and 5 mM β-mercaptoethanol (Sigma-Aldrich, Oakville, ON). Lipopolysaccharide from Escherichia coli (LPS; Sigma-Aldrich) was used as activation stimulus for DCs and B cells. Flow cytometry buffer (FACS buffer) was prepared by supplementing phosphate buffered saline (PBS; Fisher Scientific) with 2% FBS, 0.5% ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich), and 0.1% sodium azide (Sigma-Aldrich). Red blood cell lysis buffer was prepared with 150 mM ammonium chloride (NH₄Cl), 10 mM potassium bicarbonate (KHCO₃) and 100 μM EDTA in distilled water (dH₂O; all purchased from Sigma-Aldrich).

To evaluate DC phenotype by flow cytometry, the following monoclonal
antibodies were used according to manufacturer’s protocol: purified α-CD16.1/32.1 (Fc Block), allophycocyanin-α-CD11c (eBioscience, San Diego, CA), Alexa Fluor 488-MHC II (I-A/I-E) (Biolegend, San Diego, CA), PE-α-CD86 and PE-α-CD40 (BD Bioscience, Mississauga, ON). Isotype controls included PE-Rat IgG2a, allophycocyanin-Armenian hamster IgG1κ (eBioscience), and Alexa Fluor 488-Rat IgG2bk (Biolegend). To assess cell survival, 7-aminoactinomycin D viability staining solution (7-AAD; eBioscience) was used. T cell surface expression of the IL-2 receptor (CD25) was assessed by flow cytometry using an allophycocyanin-α-CD25 monoclonal antibody and an allophycocyanin-Rat IgG1 isotype control (eBioscience).

Western blot antibodies against phospho-Akt (Ser-473; polyclonal) and total Akt (polyclonal) were purchased from Cell Signaling Technology Inc (Danvers, MA). Actin, phospho-ERK (Tyr-204; clone E-4) and total ERK (clone K-23) were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA). Stock antibodies were diluted 1:1000 in Tris-buffered saline (20 mM Tris-HCl [pH 7.6], 200 mM NaCl) and 0.05% Tween-20 (TBST; all purchased from Bio-Shop Canada, Burlington, ON) containing either 5% fat-free milk or 5% bovine serum albumin (BSA; Sigma-Aldrich), according to manufacturer’s instruction.

2.3 GENERATION OF BONE MARROW-DERIVED DCs

Mature DCs were cultured from bone marrow of C57BL/6 mice as previously described (Al-Alwan et al., 2001, Lutz et al., 1999). Briefly, bone marrow cells were isolated from the femur and tibia and red blood cells were subjected to lysis. Following neutralization of the lysis buffer with FBS, bone marrow cells were cultured at 1 x 10^6
cells per well in complete RPMI 1640 medium with 20 ng/mL recombinant mouse GM-CSF (R&D Systems) in a six-well plate. Complete medium with 20 ng/mL GM-CSF was added to the cells on day 3 (d3). On d6, non adherent cells were washed and cultured in fresh complete RPMI 1640 medium containing 10 ng/mL GM-CSF.

DCs were harvested on d7 and activated with 500 ng LPS in the presence or absence of adenosine diphosphate (ADP; Bio-Data Corporation, Horsham, PA) or adenosine 5’-β-thio diphosphate (ADPβS; Sigma-Aldrich) for 24 h. Day 8 DCs were washed and analyzed using a BD FACSCalibur (Mississauga, ON) flow cytometer for MHC II and co-stimulatory molecule expression. In other experiments, DCs were used to activate T cells. For all assays, ‘control’ indicates the LPS alone group.

2.4 ISOLATION OF SPLENIC T CELLS

Pan T cells, CD4+, and CD8+ T cells and were isolated from the spleens of C57BL/6 mice using the appropriate MACS negative isolation systems (Miltenyi Biotech, Auburn, CA) according to the manufacturer’s protocol. Briefly, spleens were homogenized in PBS and red blood cells were lysed. Using negative antibody selection, T cells were purified with a MACS immunocolumn. Where indicated, CD4+ T cells were isolated from the spleens of OT-II mice using the same method.

2.5 DC-INDUCED T CELL PROLIFERATION

Purified OT-II CD4+ T cells were labeled with 5 μM carboxyfluorescein diacetate succinimidy ester (CFSE; Invitrogen) in PBS for 7 min at 37°C. Excess dye was
inactivated by FBS and T cells were washed with complete RPMI 1640 medium. Labeled T cells were co-cultured with d8 DCs at a 10:1 ratio in round-bottom 96-well plates in the presence of 100 nM chicken ovalbumin OVA peptide (amino acids 323-339; Sigma-Aldrich) and increasing concentrations of ADP or ADP\(\beta\)S. Where indicated, DCs were pre-treated with ADP or ADP\(\beta\)S on d7 and washed prior to co-culture with T cells and OVA peptide. Following 72 h culture at 37°C, 5% CO\(_2\), T cells were harvested and proliferation was measured by CFSE dilution using a BD FACScalibur flow cytometer.

Following analysis of CFSE dilution assay with FCS Express software (DeNovo Los Angeles, CA), the percentage of T cell responders was calculated as previously described (Wells et al., 1997). Briefly, a T cell generates \(2^n\) daughter cells when dividing \(n\) times. The number of cells in each division peak (as determined by fluorescence intensity) was normalized by dividing the fluorescence intensity by \(2^n\). The percentage of T cell responders was determined by comparing the total normalized number of responding cells in each fluorescence peak to the left of the parent (or non-responder) peak to the normalized number of all cells (including responders and non-responders).

### 2.7 T cell Activation and Proliferation

CD\(4^+\) and CD\(8^+\) T cells were isolated and labeled with CFSE as described above. Cells were cultured with \(\alpha\)-CD3, \(\alpha\)-CD28 coated microbeads (Dynabeads Mouse T-Activator, Invitrogen) in a 2.5:1 ratio for 48 h at 37°C in the presence of ADP or ADP\(\beta\)S. Following incubation, supernatants were collected for subsequent cytokine measurement by ELISA. Proliferation was assessed, as described above, by CFSE dilution.
Where indicated, 10 U of recombinant mouse IL-2 (Peprotech, Rocky Hill, NJ) or 25% supernatant from untreated microbead-activated pan T cells was added to CD4+ T cell cultures. Proliferation was assessed by CFSE dilution assay at 48 h as described above.

2.8 Flow Cytometry

Following 24 h pre-treatment of DC with ADP or ADPβS, 2.5 x 10^5 cells were fluorescently stained for surface expression of MHC-II, CD40 and CD86. Briefly, cells were incubated with Fc block for 30 min at 4°C before staining with specific antibodies (or the respective isotype controls) for 30 min. During the last 10 min of incubation, 7-AAD was added to each group for viability assessment. Cells were washed twice in FACS buffer and then re-suspended in FACS buffer or 2% [w/v] paraformaldehyde (PFA, Sigma Aldrich) for flow cytometry analysis. The same method was used to assess CD25 surface expression on CD4+ and CD8+ T cells following 24 h activation with microbeads.

2.9 In vitro Migration Assays

Transmigration assays were performed using the transwell system (Corning, Corning, NY) with 5.0 µm pore inserts. Membranes were coated with 0.5% [w/v] BSA (Sigma-Aldrich) for 2 h at 37°C. Inserts were washed with PBS and complete RPMI 1640 medium (600 µl) was added to each of the lower wells. In some wells, 500 ng/mL CCL19 (R&D Systems) was added to the lower chamber to serve as a chemoattractant, with or
without 50 μM ADP or 50 μM ADPβS. Naïve CD4+ T cells (5.0 x 10^5) were added to the top chamber of each insert and incubated for 4 h at 37°C. Cells that migrated to the bottom chamber were harvested and counted using a hemocytometer. All migration conditions were done in duplicate and each experiment was repeated 3 times.

2.10 CYTOTOXIC T LYMPHOCYTE (CTL) ACTIVITY

Isolated pan T cells were activated by co-culture with syngenic bone marrow derived DC and 5 ug/mL α-TCRβ (eBioscience) for 72 h. Following activation, T cells were cultured in 96-well round bottom plates with 1 ug/mL α-TCRβ and 1 x 10^4 P815 tumour cell targets (kindly provided by Dr. D. Hoskin) labeled with 5 μCi/mL methyl tritiated thymidine ([³H]TdR; MP Biomedicals, Montreal, QC) at various effector to target ratios. Plates were incubated at 37°C for 5 h, after which contents of each well were harvested onto fibreglass filter mats with a Titretek® Cell Harvester (Skatron Instruments, Sterling VA). [³H]-TdR release from lysed target cells was measured using a Beckman LS6000IC liquid scintillation counter (Beckman Coulter Inc, Mississauga, ON). Specific lysis was calculated using the formula: % specific lysis = ([³H]TdR_{CPM in targets} - [³H]TdR_{CPM in effector+targets})/ ([³H]TdR_{CPM in targets}) x 100%.

2.11 CYTOKINE ANALYSIS

Cytokines in T cell supernatants harvested from bead-induced proliferation assays were analyzed by Enzyme-Linked Immunosorbent Assay (ELISA) using commercially available kits from R&D systems. CD4+ and CD8+ T cell supernatants were analyzed for
IL-2 and IFN-γ production, respectively. Briefly, T cell supernatants and standards were added in triplicate to an EIA/RIA 96-well flat-bottom plate, pre-coated with α-IL-2 or α-IFN-γ capture antibodies. Following 2 h incubation at room temperature, wells were washed and biotinylated detection antibodies were added for 2 h. Wells were washed and Streptavadin-horseradish peroxidase (HRP) solution was added and incubated at room temperature for 20 min in the dark. Following an additional wash, substrate solution was added and plates were incubated at room temperature for 20 min, shielded from the light. An H₂SO₄ stop solution was added and absorbance at 450 nm was measured using an Asys Expert 96 colorimetric microplate reader (Montreal Biotech, Montreal, QC).

2.12 PREPARATION OF CELL LYSATES AND WESTERN BLOT ANALYSIS

CD4⁺ T cells were activated with microbeads as described above and incubated for the indicated times in 1.5 mL Eppendorf tubes. Cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 50 mM Na₂HPO₄ 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 5 mM EDTA, 5 mM ethylene glycol-bis(β-aminoethyl ether)-N, N’, N’’-tetraacetic acid [EGTA]) containing freshly added protease and phosphatase inhibitors; 5 μg/mL leupeptin, 5 μg/mL pepstatin, 10 mM NaF, 1 mM phenylmethyl sulfonyl fluoride (all purchased from Sigma-Aldrich), 1 mM dithiothreitol (DTT; Bio-Shop Canada, Burlington, ON), 100 μM Na₃VO₄ (EMD Chemicals, Gibbstown, NJ), 10 μM phenylarsine oxide, and 10 μg/mL aprotinin (Sigma-Aldrich). Samples were incubated on ice for 10 minutes and clarified by centrifugation at 20 000g for 10 min. Total cell protein was collected and quantified by colorimetric assay using Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories Inc, Mississauga, ON).
Protein levels were equalized between samples and samples were denatured in sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) loading buffer: 200 mM Tris-HCl [pH 6.8] (Fisher Scientific), 30% glycerol (VWR, Mississauga, ON), 6% [w/v] SDS (Bio-Shop Canada, Burlington, ON), 15% [v/v] β-mercaptoethanol (Sigma-Aldrich) and 0.01% [w/v] bromophenol blue (Sigma-Aldrich). Each sample was heated to 95°C for 5 min and frozen at -80°C until further use.

Pre-stained protein ladders (Bio-Rad Laboratories) and protein samples were resolved on 12% Tris-HCl acrylamide gels (containing 375 mM Tris-HCl [pH 8.8], 0.1% SDS, 0.1% ammonium persulfate (APS) and 0.15% N, N', N', N'-tetramethylethylenediamine (TEMED)) with a 4% acrylamide stacking gel (containing 125 mM Tris-HCl [pH 6.8], 0.1% SDS, 0.1% APS and 0.3% TEMED). Gels were subjected to electrophoresis at 200 V for 1 h in SDS-PAGE running buffer (20 mM Tris-HCl [pH 8.3], 200 mM glycine and 0.1% SDS; all purchased from Bio-Shop Canada) and transferred to nitrocellulose membranes using the iBlot® dry blotting system (Invitrogen). Nitrocellulose membranes were incubated in blocking solution containing TBST with 5% fat free milk for 1 h at room temperature. Membranes were incubated with the appropriate primary antibody solution overnight at 4°C. Following extensive washing with TBST, membranes were incubated with the appropriate HRP-conjugated secondary antibody (diluted 1:1000 in TBST with 5% fat-free milk) for 1 h at room temperature. Membranes were reacted with enhanced chemiluminescence (ECL) reagents (GE Healthcare; Baie D’Urfe, QC), exposed to X-ray film (Fuji, Sci-Med Inc Truro, NS) and processed in a Kodak X-OMAT 1000A automated X-ray developer. Where appropriate, membranes were stripped using stripping buffer (62.5 mM Tris, 2% SDS,
100.1 mM β-mercaptoethanol (Sigma-Aldrich), pH 6.7) and re-probed for actin (to control for protein loading) or for total protein expression when phosphorylated proteins were examined. Protein bands were quantified by densitometry using AlphaEase ® FC software (Cell Biosciences, Santa Clara, CA).

### 2.13 RNA EXTRACTION, cDNA SYNTHESIS AND RT-PCR

Day 8 DCs, naïve CD4+ and CD8+ T cells, B cells and brain tissue isolated from C57BL/6 mice were analyzed by reverse transcription- polymerase chain reaction (RT-PCR) for expression of purinergic receptor (P2Y1, P2Y12 and P2Y13) mRNA. Cells (2x10^6) were lysed in TRIzol reagent (Invitrogen). Total RNA was isolated by chloroform extraction and isopropanol precipitation. RNA was washed in ethanol and resuspended in diethyl pyrocarbonate water and treated with 2.5 U of RNase-free DNase (Promega, Madison, WI) at 37°C for 30 min to eliminate potential DNA contamination. DNase was subsequently inactivated by incubation with 20 mM EGTA for 10 min at 65°C. RNA quantification and purity was assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific), with an acceptable A_{260nm}/A_{280nm} ratio being >1.8. SuperScript first strand synthesis was used to reverse-transcribe total RNA into first strand cDNA. Briefly, 2 μg RNA was combined with 50 μM oligo(dT) and 10 mM dNTP mix for 5 min at 65°C. A cDNA synthesis mix was prepared by combining 5X First Strand buffer, 0.1 M DTT, 40 U/μL RNaseOUT, and 200 U/μL SuperScript III reverse transcriptase. The synthesis mix was added to the RNA mixture for 50 min at 50°C, followed by heat inactivation of the enzyme at 70°C for 15 min. All RT-PCR reagents were purchased from Invitrogen.
To amplify cDNA, the Platinum *Taq* DNA Polymerase system (Invitrogen) was used. Primers used for amplification of purinergic receptor cDNA are listed in Table 1. Glutaraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified to control for cDNA integrity. Briefly, cDNA was combined with 10 μM of the appropriate forward and reverse primers, 10X PCR buffer, 10 mM dNTP mixture, 50 mM Mg$^{2+}$ and 2 U Platinum *Taq* polymerase. Volume was adjusted to 50 μL with water. The PCR product was amplified using a Biometra® T-gradient thermocycler (Whatman Canada Ltd, Toronto, ON) with the following cycling conditions: 94°C for 2 min; 36 cycles of 94°C for 30 sec, 54°C for 45 sec, 72°C for one min; and a final elongation step for 10 min at 72°C. Products were resolved on a 2% agarose gel containing 0.5 μg/mL ethidium bromide (Sigma-Aldrich) by electrophoresis at 120 V for 40 min. Nucleic acid bands were visualized by UV light exposure, using the Alpha Innotech Gel Imaging System (Protein Simple, Santa Clara, CA).

### 2.14 B CELL ISOLATION AND PROLIFERATION

B cells were isolated from spleens of C57BL/6 mice using the MACS negative isolation system (Miltenyi Biotech), according to the manufacturer’s protocol. B cells were cultured at 1x10^5 cells/well in 96-well round bottom plates, activated with 5 μg LPS and treated with ADP or ADPβS for 72 h at 37°C. For the last 18 h of culture, cells were pulsed with 0.5 μCi of [³H]-TdR. At the end of the incubation period, cells were harvested as previously described. Proliferation was directly correlated to [³H]-TdR incorporation by counts per minute (CPM), which was measured using a Beckman LS6000IC liquid scintillation counter. Proliferation was normalized to LPS alone group,
which is indicated as ‘control’.

### 2.15 Statistical Analysis

Experiments were repeated as indicated and error was expressed as standard deviation (SD) or standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc; La Jolla, CA). Data were analyzed using one-way ANOVA. To calculate p-values for differences between groups, the Student-Newman Keuls (for multiple comparisons) post-test was used. Results were considered to be statistically significant when \( p < 0.05 \), denoted as * \( p < 0.05 \); **, \( p < 0.01 \); ***, \( p < 0.001 \). Treatment groups were compared to the untreated control, unless otherwise indicated.
Table 1 – Primers used for RT-PCR analysis of DC and lymphocyte mRNA.

<table>
<thead>
<tr>
<th>cDNA Target</th>
<th>Primer Sequence</th>
<th>Expected product size (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2Y₁ (Laplante et al., 2010)</td>
<td>Forward: 5’-CCGAAGATCCAGTCAGTCTTGTTGAAGTAG-3’ Reverse: 5’-ACAGTACTGTCGCCCTCAACTGCAGCAGTTT-3’</td>
<td>300</td>
</tr>
<tr>
<td>P2Y₁₂ (Kronlage et al., 2010)</td>
<td>Forward: 5’-CCGGAGACACTCATATCCTTC-3’ Reverse: 5’-GCCCAAGATGACAACAGAAAG-3’</td>
<td>500</td>
</tr>
<tr>
<td>P2Y₁₃ (Kronlage et al., 2010)</td>
<td>Forward: 5’-ATTCGTGGGTTGAGCTAGTAA-3’ Reverse: 5’-ATCAGGGACCAGACGGAAT-3’</td>
<td>525</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward: 5’-CGTCCCGTAGACAAAATGGT-3’ Reverse: 5’-TTGATGGCAACAATCTCCAC-3’</td>
<td>110</td>
</tr>
</tbody>
</table>
CHAPTER 3: RESULTS

3.1 DENDRITIC CELLS EXPRESS PURINERGIC RECEPTOR mRNA

Adenosine diphosphate (ADP) and other extracellular adenine nucleotides act through interaction with G protein-coupled purinergic receptors (P2Y receptors). Specifically, ADP acts through P2Y$_1$, P2Y$_{12}$ and P2Y$_{13}$ (Webb et al., 1993, Zhang et al., 2002, Hollopeter et al., 2001). While these three receptors are highly expressed in brain tissue (Burnstock and Knight, 2004), expression of P2Y$_1$, P2Y$_{12}$ and P2Y$_{13}$ on specific immune cells remains unclear. Initially, I sought to determine if DCs and T cells expressed the ADP receptors. By RT-PCR, DCs were found to express moderate levels of P2Y$_1$, P2Y$_{12}$ and P2Y$_{13}$ mRNA, while naïve CD4$^+$ and CD8$^+$ T cells only expressed mRNA for P2Y$_1$ (Figure 2).

3.2 ADP AND ADPβS INHIBIT DC-INDUCED CD4$^+$ T CELL PROLIFERATION

DCs are professional APCs that induce activation and proliferation of naïve CD4$^+$ T cells by presentation of peptide in the context of MHC-II with co-stimulation by CD40 and CD86 (Banchereau and Steinman, 1998, Fujii et al., 2004). Induction of proliferation of naïve CD4$^+$ T cells by DCs in vitro was assessed to determine if ADP altered DC function. I used the OT-II mouse system for this assay, in which a transgenic TCR on CD4$^+$ T cells is engineered to recognize OVA in the context of class II MHC (Barnden et al., 1998). LPS-activated DCs were co-cultured with naïve, CFSE-labeled OT-II CD4$^+$ T cells, 100 nM OVA and increasing concentrations of ADP for 72 h. Proliferation of T cells was measured by CFSE-dilution, in which a leftward progression of peaks indicates
sequential rounds of proliferation. ADP was found to inhibit CD4+ T cell proliferation in a dose-dependent manner, with a significant decrease observed at 50 μM ADP, when compared to the stimulated control (Figure 3). However, since DC express high levels of ectonucleotidases CD39 and CD73, ADP can be rapidly degraded to AMP and adenosine (Deaglio and Robson, 2011). To resolve this, a stable non-hydrolysable ADP analogue (ADPβS) was also used. CD4+ T cell proliferation assays were performed as above, replacing ADP with ADPβS. Again, a dose-dependent decrease in CD4+ T cell proliferation was observed in the presence of ADPβS, when compared to the stimulated control, with significant inhibition at 50 μM ADPβS (Figure 4). The inhibition of proliferation observed with ADPβS appeared to be greater than that observed when ADP was present.

3.3 DC EXPRESSION OF MHC-II AND CO-STIMULATORY MOLECULES ARE UNCHANGED BY ADP AND ADPβS TREATMENT

Since DC-induced CD4+ T cell proliferation was inhibited by both ADP and ADPβS, DC surface expression of MHC-II and co-stimulatory molecules, CD40 and CD86, was assessed. Expression of these molecules on DCs is up-regulated following an activation stimulus, such as the TLR4 agonist LPS (Agrawal et al., 2003). On d7 of culture, DCs were treated for 24 h with LPS and increasing concentrations of ADP or ADPβS. By flow cytometry, the percentage of DCs positively expressing the molecules, as well as the mean fluorescence intensity (MFI) of expression was determined. DC surface expression of MHC-II, CD40 and CD86 was unchanged following treatment with 20 μM and 50 μM ADP when compared to LPS alone control (Figure 5). Similarly,
expression of these markers was unchanged with ADPβS treatment (Figure 6). ADP and
ADPβS were found to be non-toxic to DCs by 7-AAD viability staining (Figure 7).

3.4 PRE-TREATMENT OF DC WITH ADP OR ADPβS DOES NOT ALTER DC-
INDUCED CD4⁺ T CELL PROLIFERATION

Since treatment of DCs with ADP or ADPβS did not alter expression of MHC-II
and co-stimulatory molecules, but DC-induced proliferation of naïve CD4⁺ T cells was
diminished, the observed inhibitory effect may not be related to DCs. To elucidate which
cells ADP and ADPβS were acting on, DCs were pre-treated with increasing
concentrations of each nucleotide and LPS for 24 h before washing and co-culture with
naïve OT-II CD4⁺ T cells and 100 nM OVA. Measuring T cell proliferation by CFSE-
dilution as before, ADP pre-treatment was not found to alter DC-induction of CD4⁺ T
cell proliferation at concentrations up to 50 μM when compared to LPS-stimulated
control (Figure 8). Similarly, pre-treatment of DCs with ADPβS did not alter DC-
induction of T cell proliferation at the tested concentrations (Figure 9).

3.5 ADP AND ADPβS DECREASE CD4⁺ T CELL PROLIFERATION

Since pre-treatment of DCs with ADP or ADPβS did not alter T cell proliferation,
the observed inhibition may be T cell-specific. T cell stimulation involves CD3-mediated
signaling following TCR engagement by MHC-II, and binding of CD28 by CD86
(Hugues, 2010, Liwski et al., 2006). To investigate the effect of ADP and ADPβS on T
cells, α-CD3, α-CD28 microbeads were used as an activating agent. Naïve CD4⁺ T cells
were isolated from C57/BL6 mice, CFSE-labelled, and cultured in vitro with coated microbeads and increasing concentrations of ADP. By CFSE-dilution, 50 μM ADP was found to significantly decrease CD4+ T cell proliferation after 48 h, when compared to control activated cells (Figure 10). When T cells were activated in the presence of ADPβS, all concentrations tested were found to significantly inhibit proliferation by CFSE-dilution when compared to the stimulated control (Figure 11). To ensure that the decreased proliferation observed was not due to toxicity of ADP or ADPβS on T cells, 7-AAD viability staining was used. When naïve CD4+ T cells were activated for 24 h in the presence of 50 μM ADP or ADPβS, treatments were found to be non-toxic to cells (Figure 12).

3.6 IL-2 SECRETION IS DECREASED IN ADP- AND ADPβS-TREATED CD4+ T CELLS

Following signal 2 of activation, T cells increase production and secretion of the cytokine IL-2. IL-2 binds the high-affinity IL-2 receptor, CD25, in an autocrine manner to promote proliferation, growth and survival of T cells (Smith, 1980). Without secretion and stimulation by IL-2, T cells enter a state of anergy (reviewed by Wells, 2009). Since the presence of ADP and ADPβS during activation inhibited CD4+ T cell proliferation in a dose-dependent manner, I wanted to determine if IL-2 secretion was decreased as well. Supernatants from CD4+ T cells activated in the presence of increasing concentrations of ADP or ADPβS were collected and IL-2 secretion was measured by ELISA. IL-2 secretion by activated T cells was found to be significantly decreased in the presence of
20 μM and 50 μM ADP, when compared to the stimulated control which had a maximum detected concentration of 900 pg/mL IL-2 (Figure 13A). Similarly, IL-2 secretion was significantly inhibited when T cells were activated in the presence of each tested concentration of ADPβS when compared to the stimulated control (C\text{max} of 1200 pg/mL IL-2; Figure 13B).

3.7 PROLIFERATION OF CD4\textsuperscript{+} T CELLS CANNOT BE RESCUED BY EXOGENOUS IL-2

The reduced secretion of IL-2 by CD4\textsuperscript{+} T cells activated in the presence of ADP or ADPβS may have accounted for the reduced proliferative ability that was observed. In an attempt to restore proliferation, 10 U (approximately 2000 pg/mL) of exogenous recombinant mouse IL-2 were added to T cell cultures during activation. As before, when CD4\textsuperscript{+} T cells were activated by microbeads in the presence of 50 μM ADP, proliferation was found to be significantly inhibited. When IL-2 was added to 50 μM ADP-treated cultures, T cell proliferation was also significantly inhibited, with no significant difference in proliferation between ADP and ADP + IL-2 treated groups (Figure 14). When CD4\textsuperscript{+} T cells were activated in the presence of 50 μM ADPβS, with or without IL-2, proliferation was significantly inhibited when compared to the stimulated control. There was no significant difference between ADPβS and ADPβS + IL-2 groups (Figure 15).
3.8 Proliferation of CD4+ T Cells Cannot Be Rescued by Activated T Cell Supernatant

Although IL-2 provides growth and survival signals to T cells through CD25, other cytokines and growth factors present in the T cell milieu during activation can promote proliferation as well (He et al., 1997, Malek et al., 2001). Since ADP and ADPβS decrease IL-2 secretion by T cells, it is possible that other signals could also be missing from the T cell environment, which could contribute to the decreased proliferation that was observed. Pan T cells were activated in vitro for 48 h and supernatants were collected. Naïve CD4+ T cells were activated as before in the presence of 50 μM ADP (Figure 16) or 50 μM ADPβS (Figure 17), with or without a 1:4 dilution of activated T cell supernatant. For both ADP and the analogue, a significant decrease in proliferation was observed when compared to the stimulated control, with no significant difference observed between nucleotide and nucleotide with supernatant groups.

3.9 Surface Expression of CD25 Is Inhibited by ADP and ADPβS on CD4+ T Cells

The addition of exogenous IL-2 or activated T cell supernatant did not rescue proliferation of CD4+ T cells, indicating that ADP and ADPβS may be affecting a signaling pathway involved in T cell activation. Since CD25 is quickly up-regulated on the surface of T cells and high expression is required to perpetuate T cell activation and proliferation (Smith, 1980, Nelson and Willerford, 1998), I wanted to investigate whether ADP or ADPβS were preventing expression of CD25 on CD4+ T cells following
activation. To determine surface expression of CD25, CD4+ T cells were activated by microbeads in the presence or absence of 50 μM ADP or ADPβS for 24 h. By flow cytometry, ADP and ADPβS were found to significantly reduce surface expression of CD25, when compared to the stimulated T cell control (Figure 18).

3.8 Phosphorylation of ERK1 and Akt May Be Inhibited by ADP and ADPβS

Since proliferation, IL-2 secretion and CD25 expression were all inhibited following CD4+ T cell activation in the presence of ADP or ADPβS, I decided to investigate the activity of signaling molecules which may be involved in T cell activation. ERK1 and ERK2 are serine threonine kinases involved in cell-cycle progression and promotion of IL-2 expression (Rauscher et al., 1988, Whitehurst and Geppert, 1996). Similarly, Akt is a central signaling molecule implicated in cell survival, growth, and cell cycle progression (Burr et al., 2001, Kane et al., 2001). The phosphorylation status (increased phosphorylation indicates increased activity) was investigated for these molecules by western blot analysis. Phosphorylation of ERK1 (44 kDa, upper band), but not ERK2 (42 kDa, lower band), appeared to be decreased 5 min after CD4+ T cells were activated in the presence of ADP or ADPβS, when compared to activated CD4+ T cells (Figure 19). Likewise, Akt phosphorylation also appeared to be decreased in the presence of ADP or ADPβS, 60 min following coated microbead activation of CD4+ T cells, when compared to the stimulated control (Figure 20).
3.9 Migration of Naïve CD4⁺ T Cells is Inhibited In Vitro by ADP and ADPβS

ADP is present at increased concentrations at sites of inflammation, due to degradation of ATP that is released from necrotic cells (Di Virgilio et al., 2001). Also, ADP could be released at sites of T cell activation following metastasis of cancer cells to the draining lymph node (Aymeric et al., 2010). Since ADP inhibits CD4⁺ T cell proliferation, and may contribute to decreased ERK and Akt signaling, I wanted to investigate if ADP could inhibit migration of T cells to inflamed tissues. An in vitro transmigration assay was performed using the Transwell system, in which naïve T cells were placed on top of a 5.0 μm pore membrane and allowed to migrate toward the chemokine CCL19, with or without 50 μM ADP or ADPβS, in the bottom chamber. When compared to the transmigration of T cells to CCL19 alone, transmigration toward ADP and ADPβS was significantly inhibited (Figure 21).

3.10 Proliferation of CD8⁺ T Cells is Unchanged by ADP and ADPβS

Naïve CD8⁺ T cells express mRNA for the receptor P2Y₁ (Figure 2), and as such, may have altered activity in the presence of ADP or ADPβS. CD8⁺ T cells were therefore isolated and activated in vitro with α-CD3 and α-CD28 coated microbeads. In the presence of ADP or ADPβS, there were no significant decreases in ADP- or ADPβS-treated CD8⁺ T cell proliferation by CFSE-dilution, when compared to stimulated control (Figure 22 and Figure 23, respectively). When CD8⁺ T cells were exposed to ADP or
ADPβS, cells appeared to go through fewer rounds of proliferation, although this trend did not reach significance. Both nucleotides were found to be non-toxic to cells following 24 h treatment (Figure 24).

3.11 Surface Expression of CD25 is Significantly Reduced on CD8+ T Cells by ADP and ADPβS

As with CD4+ T cells, CD25 is up-regulated on CD8+ T cells after activation, to enhance autocrine interaction with IL-2 (Mescher et al., 2006). Surface expression of CD25 on CD8+ T cells activated in the presence of 50 μM ADP or ADPβS was measured by flow cytometry. ADP and ADPβS were found to significantly reduce surface expression of CD25 on CD8+ T cells, when compared to stimulated T cell control (Figure 25).

3.12 Secretion of IFN-γ by CD8+ T Cells Is Inhibited by ADP and ADPβS, But Cytotoxic Ability Is Unchanged

Important in type 1 effector CD8+ T cell responses is the production of IFN-γ, which can promote anti-viral immune responses, as well as CTL activity (Sercan et al., 2006). CD8+ T cells were activated in the presence of increasing concentrations of ADP or ADPβS and supernatants were collected to measure IFN-γ secretion by ELISA. All tested concentrations of ADP and ADPβS were found to significantly inhibit IFN-γ secretion when compared to stimulated control (maximum detected IFN-γ concentration of 700 pg/mL; Figure 26A and 26B for ADP and ADPβS, respectively).
Cytotoxic T cells were generated *in vitro* by activation of pan T cells with DC and α-TCR-β, in the presence of 50 μM ADP or ADPβS. Following incubation with α-TCR-β and P815 mastocytoma cells, which are allogeneic to C57BL/6, targeted lysis by CTLs was assessed by release of [³H]-TdR. At the tested effector: target ratios, killing activity of CTLs generated in the presence of ADP or ADPβS was found to be comparable to stimulated CTLs with 30-40% specific lysis at the effector: target ratio of 50:1 (Figure 27).

### 3.13 Proliferation of B Cells is Increased in the Presence of ADP and ADPβS

In adaptive immunity, B cells are important for not only antibody production, but for enhancing DC-mediated T cell activation in secondary immune responses. In particular, B cells are able to proliferate rapidly and allow for antigen spreading during an immune response (reviewed by Cerutti et al., 2011). By RT-PCR, B cells express ADP receptor P2Y₁₃ mRNA, albeit at a low level (Figure 28). To determine if B cell proliferative ability was affected by ADP or ADPβS, B cells were activated by LPS in the presence of increasing concentrations of the nucleotides. By [³H] TdR incorporation (measured by CPM), proliferation of B cells was unchanged by ADP, compared to cells treated with LPS alone (Figure 29A). Proliferation of B cells was significantly increased when activated with LPS in the presence of 50 μM ADPβS (Figure 29B).
Figure 2. Expression of ADP purinergic receptor mRNA in DC, CD4+ and CD8+ T cells. RNA was extracted from mouse DC, CD4+ and CD8+ T cell lysates. By RT-PCR, mRNA expression of the ADP receptors P2Y1, P2Y12, and P2Y13 was assessed in each cell type. Brain mRNA was used as a positive control for all receptors. Results are representative of n=2.
Figure 3. ADP causes a decrease in DC-induced CD4+ T cell proliferation. OTII CD4+ T cells were cultured for 72 h in the presence of syngeneic, LPS-activated C57BL/6 DC, 100 nM OVA peptide and increasing concentrations of ADP. Proliferation of T cells was measured by flow cytometry using a CFSE dilution assay. A representative histogram of T cell proliferation in the presence of ADP is provided (A), with cumulative percent responders calculated and shown in (B). Results are expressed as mean +/- SD of at least 3 experiments. (*p<0.05)
A

![Graph showing T cell responses to different ADP concentrations.](image)

- T cells alone
- Stimulated
- 10uM ADP
- 20uM ADP
- 50uM ADP

B

![Bar chart showing % CD4+ OTI T cell responders.](image)

- T cells alone
- Stimulated
- 10uM ADP
- 20uM ADP
- 50uM ADP

+ LPS-activated DC
Figure 4. ADPβS causes a decrease in DC-induced CD4+ T cell proliferation. OTII CD4+ T cells were cultured for 72 h in the presence of syngeneic, LPS-activated C57BL/6 DC, 100 nM OVA peptide and increasing concentrations of ADPβS. Proliferation of T cells was measured by flow cytometry using a CFSE dilution assay. A representative histogram of T cell proliferation in the presence of ADPβS is provided (A), with cumulative percent responders calculated and shown in (B). Results are expressed as mean +/- SD of at least 3 experiments. (**p<0.001)
A

![Graph showing proliferation of T cells alone and stimulated with different concentrations of ADP.]

B

![Bar graph showing the percentage of CD4+ OTII T cell responders with standard errors.]

% CD4^+ OTII T cell responders

T cells alone, Stimulated, 10uM ADPβS, 20uM ADPβS, 50uM ADPβS

+ LPS-activated DC
Figure 5. ADP does not affect DC MHC-II or co-stimulatory molecule expression.

DCs were derived in vitro from bone marrow precursors in the presence of GM-CSF. On d7, DC were treated with activation stimulus LPS in the presence or absence of increasing concentrations of ADP. Flow cytometric analysis of MHC-II, CD40 and CD86 surface expression was performed on d8. Percent positive cells relative to isotype control (A), and mean fluorescence intensity (B), were calculated. Results are expressed as mean +/- SD of at least 3 independent experiments.
Figure 6. ADPβS does not affect DC MHC-II or co-stimulatory molecule expression. DCs were cultured in vitro from bone marrow precursors in the presence of GM-CSF. On d7, DC were treated with maturation stimulus LPS in the presence or absence of increasing concentrations of ADPβS. Flow cytometric analysis of MHC-II, CD40 and CD86 surface expression was performed on d8. Percent positive cells relative to isotype control (A), and mean fluorescence intensity (B), were calculated. Results are expressed as mean +/- SD of at least 3 independent experiments.
Figure 7. Treatment of DC with ADP or ADPβS does not cause cell death. DCs were cultured in vitro from bone marrow precursors in the presence of GM-CSF. On d7, DCs were treated with maturation stimulus LPS in the presence or absence of ADP or ADPβS for 24 h. 7-AAD viability staining was used to measure cell death by flow cytometry. Representative scatter plots (A) are shown, with cumulative results calculated and shown in (B). Results are expressed as mean +/- SD of at least 3 experiments.
A

LPS alone + 50uM ADP + 50uM ADPβS

B

% live cells

LPS alone + 50uM ADP + 50uM ADPβS
Figure 8. Pre-treatment of DC with ADP does not alter DC-induced CD4+ T cell proliferation. Day 7 DC were treated with increasing concentrations of ADP and matured with LPS for 24 h. DC were washed and cultured for 72 h with naïve OTII CD4+ T cells in the presence of 100 nM OVA peptide. Proliferation of T cells was measured by flow cytometry using a CFSE dilution assay. A representative histogram of T cell proliferation in the presence of ADP is provided (A), with cumulative percent responders calculated and shown in (B). Results are expressed as mean +/- SD of at least 3 experiments.
A

T cells alone

Stimulated

10uM ADP

20uM ADP

50uM ADP

Count

Proliferation

B

% CD4+ OTII T cell responders

T cells alone

Stimulated

10uM ADP

20uM ADP

50uM ADP

+ LPS-activated DC
Figure 9. Pre-treatment of DC with ADPβS does not alter DC-induced CD4⁺ T cell proliferation. Day 7 DC were treated with increasing concentrations of ADPβS and matured with LPS for 24 h. DC were washed and cultured for 72 h with naïve OTII CD4⁺ T cells in the presence of 100 nM OVA peptide. Proliferation of T cells was measured by flow cytometry using a CFSE dilution assay. A representative histogram of T cell proliferation in the presence of ADP is provided (A), with cumulative percent responders calculated and shown in (B). Results are expressed as mean +/- SD of at least 3 experiments.
A

![Graph showing proliferation of T cells under different conditions](image)

- **T cells alone**
- **Stimulated**
- **10uM ADPβS**
- **20uM ADPβS**
- **50uM ADPβS**

B

![Bar chart showing % CD4^+ OTII T cell responders](image)

- **T cells alone**
- **Stimulated**
- **10uM ADPβS**
- **20uM ADPβS**
- **50uM ADPβS**

+ LPS-activated DC
Figure 10. ADP decreases CD4$^+$ T cell proliferation. Naïve CD4$^+$ T cells were isolated from C57/BL6 mice and activated in vitro with α-CD3, α-CD28 coated microbeads in the presence of increasing concentrations of ADP for 48 h. Proliferation of T cells was measured by flow cytometry using a CFSE dilution assay. A representative histogram of T cell proliferation in the presence of ADP is provided (A), with cumulative percent responders calculated and shown in (B). Results are expressed as mean +/- SD of at least 3 experiments. (*p<0.05)
A

![Graph showing proliferation counts for different conditions.]

B

![Bar chart showing percentage of CD4+ T cell responders. * indicates a significant difference.]

+ α-CD3, α-CD28 beads
Figure 11. ADPβS decreases CD4+ T cell proliferation. Naïve CD4+ T cells were isolated from C57/BL6 mice and activated in vitro with α-CD3, α-CD28 coated microbeads in the presence of increasing concentrations of ADPβS for 48 h. Proliferation of T cells was measured by flow cytometry using a CFSE dilution assay. A representative histogram of T cell proliferation in the presence of ADP is provided (A), with cumulative percent responders calculated and shown in (B). Results are expressed as mean +/- SD of at least 3 experiments. (**p<0.01, ***p<0.001)
**A**

![Graph showing T cell proliferation](image)

- **T cells alone**
- **Stimulated**
- **10uM ADP β S**
- **20uM ADP β S**
- **50uM ADP β S**

**B**

![Bar chart showing percentage of CD4⁺ T cell responders](image)

- **T cells alone**
- **Stimulated**
- **10uM ADP β S**
- **20uM ADP β S**
- **50uM ADP β S**

+ α-CD3, α-CD28 beads

---

**Note:** The image contains graphs and bar charts illustrating the response of T cells to different ADP concentrations and stimulation with α-CD3, α-CD28 beads.
Figure 12. Treatment of CD4⁺ T cells with ADP or ADPβS does not cause cell death. Naïve CD4⁺ T cells were isolated from C57/BL6 mice and activated in vitro with α-CD3, α-CD28 coated microbeads in the presence of ADP or ADPβS for 24 h. 7-AAD viability staining was used to measure cell death by flow cytometry. Representative scatter plots (A) are shown, with cumulative results calculated and shown in (B). Results are expressed as mean +/- SD of at least 3 experiments.
A

Stimulated

50uM ADP

50uM ADPβS

96.93%

94.94%

96.25%

B

% live cells

0

50

100

Stimulated

50uM ADP

50uM ADPβS
Figure 13. ADP or ADPβS treatment cause decreased IL-2 secretion by CD4^+ T cells. Naïve CD4^+ T cells were isolated from C57/BL6 mice and activated in vitro with α-CD3, α-CD28 coated microbeads in the presence of increasing concentrations of ADP (A) or ADPβS (B) for 48 h. Supernatants were collected and IL-2 secretion was measured using ELISA. Maximum concentration (C_{max}) of IL-2 measured in untreated CD4^+ T cell cultures was 900 pg/mL (A) or 1200 pg/mL (B). Results were normalized to untreated controls and are expressed as mean +/- SEM of at least 3 experiments. (**p<0.01, ***p<0.001)
Figure 14. Addition of exogenous IL-2 to ADP treated CD4⁺ T cells does not rescue proliferation. Naïve CD4⁺ T cells were isolated from C57/BL6 mice and activated *in vitro* with α-CD3, α-CD28 coated microbeads in the presence of ADP and 10 U recombinant mouse IL-2 for 48 h. Proliferation of T cells was measured by flow cytometry using CFSE dilution. A representative histogram of T cell proliferation in the presence of ADP is provided (A), with cumulative percent responders calculated and shown in (B). Results are expressed as mean +/- SD of at least 3 experiments. (NS $p>0.05$, ***$p<0.001$)
A

![Graph A](image)

B

![Graph B](image)
Figure 15. Addition of exogenous IL-2 to ADPβS treated CD4+ T cells does not rescue proliferation. Naïve CD4+ T cells were isolated from C57/BL6 mice and activated in vitro with α-CD3, α-CD28 coated microbeads in the presence of ADPβS and 10 U recombinant mouse IL-2 for 48 h. Proliferation of T cells was measured by flow cytometry using CFSE dilution. A representative histogram of T cell proliferation in the presence of ADPβS is provided (A), with cumulative percent responders calculated and shown in (B). Results are expressed as mean +/- SD of at least 3 experiments. (NS p>0.05, **p<0.01, ***p<0.001)
A

![Graph showing proliferation of T cells under different conditions.]

- T cells alone
- Stimulated
- 50uM ADP β S
- 50uM ADP β S + IL-2

B

![Bar graph showing percentage of CD4+ T cell responders.]

- T cells alone
- Stimulated
- 50uM ADP β S
- 50uM ADP β S + IL-2

+ α-CD3, α-CD28 beads
Figure 16. Addition of activated T cell supernatant to ADP treated CD4+ T cells does not rescue proliferation. Naïve CD4+ T cells were isolated from C57/BL6 mice and activated in vitro with α-CD3, α-CD28 coated microbeads in 25% activated pan T cell supernatant and ADP for 48 h. Proliferation of T cells was measured by flow cytometry using CFSE dilution. A representative histogram of T cell proliferation in the presence of ADP is provided (A), with cumulative percent responders calculated and shown in (B). Results are expressed as mean +/- SD of at least 3 experiments. (NS $p>0.05$, ***$p<0.001$)
A

![Graph showing cell proliferation](image)

- **T cells alone**
- **Stimulated**
- **50uM ADP**
- **50uM ADP + supematant**

B

![Bar graph showing % CD4+ T cell responders](image)

- **T cells alone**
- **Stimulated**
- **50uM ADP**
- **50uM ADP + supematant + α-CD3, α-CD28 beads**

- NS
- ***
- ***
Figure 17. Addition of activated T cell supernatant to ADPβS treated CD4⁺ T cells does not rescue proliferation. Naïve CD4⁺ T cells were isolated from C57/BL6 mice and activated in vitro with α-CD3, α-CD28 coated microbeads in 25% activated pan T cell supernatant and ADPβS for 48 h. Proliferation of T cells was measured by flow cytometry using CFSE dilution. A representative histogram of T cell proliferation in the presence of ADPβS is provided (A), with cumulative percent responders calculated and shown in (B). Results are expressed as mean +/- SD of at least 3 experiments. (NS p>0.05, **p<0.01, ***p<0.001)
A

![Graph showing cell proliferation](image)

B

![Bar chart showing CD4+ T cell responders](image)

+ α-CD3, α-CD28 beads
Figure 18. CD25 expression is significantly reduced by treatment of CD4⁺ T cells with ADP or ADPβS. Naïve CD4⁺ T cells were isolated from C57/BL6 mice and activated in vitro with α-CD3, α-CD28 coated microbeads in the presence of ADP or ADPβS for 24 h. Surface expression of CD25 was determined by flow cytometry. Percent positive cells relative to isotype control (A), and mean fluorescence intensity (B), were calculated. Results are expressed as mean +/- SD of at least 3 independent experiments. (*p<0.05)
Figure 19: Phosphorylation of ERK1 in CD4+ T cells during activation seems to be inhibited by ADP and ADPβS. Naïve CD4+ T cells were isolated from C57/BL6 mice and activated in vitro with α-CD3, α-CD28 microbeads in the presence of ADP or ADPβS for 5 min. Protein lysates were prepared, resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blotted with the appropriate antibodies and x-ray chemiluminescence was used to detect protein presence. Western blots are representative of n=2 (A). Expression of pERK1, relative to tERK1, is shown by representative densitometric analysis (B).
**A**

<table>
<thead>
<tr>
<th></th>
<th>T cells alone</th>
<th>Stimulated</th>
<th>50uM ADP</th>
<th>50uM ADPβ3</th>
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<tr>
<td><strong>pERK1</strong></td>
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<td><strong>tERK2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Actin</strong></td>
<td></td>
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</tr>
</tbody>
</table>

**B**

![Bar chart showing relative expression of pERK1/ERK1](chart.png)
Figure 20. Phosphorylation of Akt in CD4+ T cells during activation seems to be inhibited by ADP and ADPβS. Naïve CD4+ T cells were isolated from C57/BL6 mice and activated in vitro with α-CD3, α-CD28 microbeads in the presence of ADP or ADPβS for 60 min. Protein lysates were prepared, resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were blotted with the appropriate antibodies and x-ray chemiluminescence was used to detect protein presence. Western blots are representative of n=2 (A). Expression of pAkt, relative to tAkt, is shown by representative densitometric analysis (B).
A

+ α-CD3, α-CD28 beads

<table>
<thead>
<tr>
<th></th>
<th>T cells alone</th>
<th>Stimulated</th>
<th>50uM ADP</th>
<th>50uM ADPβS</th>
</tr>
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</tr>
<tr>
<td>Actin</td>
<td></td>
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</tbody>
</table>

B

![Bar chart showing relative expression of pAkt/tAkt](image)

- T cells alone
- Stimulated
- 50 uM ADP
- 50 uM ADPβS
Figure 21. Transmigration of naïve CD4⁺ T cells is inhibited by ADP and ADPβS. Naïve CD4⁺ T cells were added to the upper chamber of a Transwell system and allowed to migrate toward RPMI supplemented with the chemokine CCL19 in the presence or absence of 50 μM ADP or ADPβS for 4 h. Cells that migrated to the bottom chamber were harvested and counted using a hemocytometer. Percent migration, relative to CCL19 alone control, was assessed. Results are expressed as mean +/- SD of 3 independent experiments. (**)p<0.01)
% migration (relative to CCL19 alone)

**Bottom well**
Figure 22. ADP does not alter CD8\(^+\) T cell proliferation. Naïve CD8\(^+\) T cells were isolated from C57/BL6 mice and activated \textit{in vitro} with \(\alpha\)-CD3, \(\alpha\)-CD28 coated microbeads in the presence of increasing concentrations of ADP for 48 h. Proliferation of T cells was measured by flow cytometry using a CFSE dilution assay. A representative histogram of T cell proliferation in the presence of ADP is provided (A), with cumulative percent responders calculated and shown in (B). Results are expressed as mean +/- SD of at least 3 experiments.
**A**

T cells alone
- Stimulated
- 10uM ADP
- 20uM ADP
- 50uM ADP

**B**

% CD8+ T cell responders
- T cells alone
- Stimulated
- 10uM ADP
- 20uM ADP
- 50uM ADP

+ α-CD3, α-CD28 beads
Figure 23. ADPβS does not alter CD8+ T cell proliferation. Naïve CD8+ T cells were isolated from C57/BL6 mice and activated in vitro with α-CD3, α-CD28 microbeads in the presence of increasing concentrations of ADPβS for 48 h. Proliferation of T cells was measured by flow cytometry using a CFSE dilution assay. A representative histogram of T cell proliferation in the presence of ADP is provided (A), with cumulative percent responders calculated and shown in (B). Results are expressed as mean +/- SD of at least 3 experiments.
Figure 24. Treatment of CD8\(^+\) T cells with ADP or ADP\(\beta\)S does not cause cell death. Naïve CD8\(^+\) T cells were isolated from C57/BL6 mice and activated in vitro with α-CD3, α-CD28 coated microbeads in the presence of ADP or ADP\(\beta\)S for 24 h. 7-AAD viability staining was used to measure cell death by flow cytometry. Representative scatter plots (A) are shown, with cumulative results calculated and shown in (B). Results are expressed as mean +/- SD of at least 3 experiments.
A

Stimulated 50uM ADP 50uM ADPβS

B

% live cells

Stimulated 50uM ADP 50uM ADPβS
Figure 25. CD25 expression is significantly reduced by treatment of CD8\(^+\) T cells with ADP or ADPβS. Naïve CD8\(^+\) T cells were isolated from C57/BL6 mice and activated \textit{in vitro} with α-CD3, α-CD28 coated microbeads in the presence of ADP or ADPβS for 24 h. Surface expression of CD25 was determined by flow cytometry. Percent positive cells relative to isotype control (A), and mean fluorescence intensity (B), were calculated. Results are expressed as mean +/- SD of at least 3 independent experiments. (*\(p<0.05\))
A

% CD25 Positive

T cells alone  Stimulated  50uM ADP  50uM ADPβS

+ α-CD3, α-CD28 beads

B

Mean Fluorescence Intensity

T cells alone  Stimulated  50uM ADP  50uM ADPβS

+ α-CD3, α-CD28 beads
Figure 26. ADP or ADPβS treatment causes decreased IFN-γ secretion by CD8^+ T cells. Naïve CD8^+ T cells were isolated from C57/BL6 mice and activated *in vitro* with α-CD3, α-CD28 coated microbeads in the presence of increasing concentrations of ADP (A) or ADPβS (B) for 48 h. Supernatants were collected and IFN-γ secretion was measured using ELISA. Maximum concentration (C_{max}) of IFN-γ measured in untreated CD8^+ T cell cultures was 700 pg/mL (A & B). Results were normalized to untreated controls and are expressed as mean +/- SEM of at least 3 experiments. (***p<0.001)
A

Stimulated 10uM ADP 20uM ADP 50uM ADP

% IFN-γ secretion

***

B

Stimulated 10uM ADPβS 20uM ADPβS 50uM ADPβS

% IFN-γ secretion

***
Figure 27. Cytotoxic T lymphocyte induction is unaffected by ADP or ADPβS treatment. Naïve pan T cells were isolated from C57/BL6 mice and activated in vitro with syngeneic bone marrow derived DC and 5 μg/mL α-TCR-β for 72 h. Following activation, T cells were co-cultured with [³H] TdR-labeled P815 tumour cells for 5 h at various effector to target ratios. Cells were harvested, [³H] TdR release was measured by scintillation and percent specific lysis was calculated. Results are expressed as mean +/- SEM of at least 3 experiments.
Stimulated
+ 50uM ADP
+ 50uM ADPβS

% specific lysis

Effector:Target Ratio
Figure 28. Expression of ADP purinergic receptor mRNA in B cells. RNA was extracted from mouse B cell lysates. By RT-PCR, mRNA expression of the ADP receptors P2Y1, P2Y12, and P2Y13 was assessed. Brain mRNA was used as a positive control for all receptors. Results are representative of n=2.
Ladder  B cell  brain

P2Y₁ (300 bp)
P2Y₁₂ (500 bp)
P2Y₁₃ (525 bp)
GAPDH (110 bp)
Figure 29. ADPβS treatment increases B cell proliferation. B cells were isolated from C57/BL6 mice and activated in vitro with 5 μg LPS in the presence of various concentrations of ADP or ADPβS for 72 h. For the last 18 h of culture, B cells were pulsed with 0.5 μCi of [3H] TdR. Cells were harvested and [3H] TdR incorporation was measured by scintillation. Proliferation was compared to LPS alone control. Results are expressed as mean +/- SEM of at least 3 experiments. (*p<0.05).
A

% proliferation by [³H] TdR incorporation

B

% proliferation by [³H] TdR incorporation
CHAPTER 4: DISCUSSION

4.1 DISCUSSION

In conditions of cellular stress or tissue injury, danger signals are released to alert and activate the immune system. ADP is generated following ectonucleotidase-mediated degradation of ATP, an endogenous danger signal (la Sala et al., 2003). While its role in inflammation has not been fully elucidated, ADP is a potent activator of platelets, indicating that it may be pro-inflammatory (Del Conde et al., 2005). It has been suggested that although ADP can activate DCs, secretion of IL-12 is decreased in response to ADP (Marteau et al., 2004), and thus DC-mediated immune responses may be affected. The objective of the current study was to determine the role of ADP in adaptive immunity. Specifically, the role of ADP in DC and T cell responses was investigated.

ADP generates cellular responses by interaction with the P2Y receptors, P2Y1, P2Y12, and P2Y13, which are expressed on DCs and lymphocytes (Burnstock and Knight, 2004, Erlinge, 2011). The concentrations of ADP used for the current study (10, 20, and 50 μM) were chosen based on the concentration required to activate platelets in vitro, according to the clinical supplier (Bio/Data Corporation). These doses are within the range expected at sites of injury (Di Virgilio et al., 2009), and have been used in other studies investigating the effect of ADP on DC function (Idzko et al., 2002, Ben Addi et al., 2010). Also, the chosen doses are greater than the half maximal effective concentration (EC50) for the ADP receptors (8 μM, 0.07 μM, and 0.06 μM, for P2Y1, P2Y12, and P2Y13, respectively) (Bours et al., 2006).
4.1.1 Effect of ADP on DC Responses

In the presence of ADP, DC-mediated CD4+ T cell activation was inhibited in a dose-dependent manner, with a significant decrease in T cell proliferation at 50 μM ADP. This result was surprising, given the hypothesis that ADP would enhance DC and T cell responses. However, as DCs express the ectonucleotidases CD39 and CD73, it was possible that the ADP was degraded rapidly in culture to adenosine, which would account for the observed suppressive effect (Deaglio and Robson, 2011, Mizumoto et al., 2002). To eliminate this possibility, a stable ADP analogue, ADPβS, was used. ADPβS is stable due to the replacement of one of the terminal oxygens on the β-phosphate with sulfur. Again, a dose-dependent decrease in DC-mediated CD4+ T cell proliferation was observed, albeit to a greater extent. The inhibitory effect of ADPβS appears to be more potent, which is likely due to its increased stability in vitro. These results suggest that the effect of ADP-mediated signaling may be different than was originally hypothesized.

Since I found that DCs express mRNA for all three known ADP receptors, I thought that it would be more likely that the effect of ADP would be specific to this cell population. However, surface expression of MHC-II and co-stimulatory molecules CD40 and CD86 was unchanged by treatment of LPS-stimulated DCs with increasing concentrations of ADP or ADPβS. These co-stimulatory molecules are normally increased on DCs following an activating stimulus (such as LPS binding TLR4), and are required to initiate an effective T cell response (Agrawal et al., 2003, Fujii et al., 2004). To date, no studies have investigated the effect of ADP or ADPβS on these DC markers specifically. One group has shown that ADPβS significantly increases surface expression of another co-stimulatory molecule, CD83, in immature DCs, although LPS-activated
cells display the same CD83 expression with or without 250 μM ADPβS (Marteau et al., 2004). Importantly, ADP and ADPβS were not toxic to DCs, even at the highest concentration tested.

Although ADP or ADPβS did not change DC expression of MHC-II, CD40 or CD86 in my model, I wanted to determine if the inhibited T cell proliferation in the presence of ADP or ADPβS was due to these nucleotides affecting DC induction of T cells. DCs stimulated in the presence of ADP or ADPβS prior to co-culture displayed a similar ability to induce proliferative responses in naïve CD4+ T cells, when compared to the control, indicating either that ADP had no effect on DCs, or that the effect may be reversible.

Full maturation of DCs involves stimulation by PAMPs or DAMPS, as well as antigen processing (Sallusto et al., 1995). Recently, Ben Addi et al. (2010) demonstrated that if ADPβS was present during DC maturation, antigen uptake was increased, correlating with enhanced DC-mediated naïve T cell proliferation. Due to my experimental design, where antigen was only present following ADP and LPS exposure, I was only investigating the effect of ADP on DC stimulation, and missing potential effects on full DC maturation. If the experimental design was adjusted in future studies, the effects of ADP we observe may correlate better with other published results.

Cytokine production by DCs was not investigated in the current study, but is important to consider as cytokines help to shape adaptive responses. DC secretion of IL-12 is inhibited in the presence of ADP (Marteau et al., 2004, Ben Addi et al., 2010), indicating that production of other cytokines may also be affected. It would be interesting to examine the cytokine profile of DCs matured in the presence of ADP to gain a better
understanding of which subset of Th cells are promoted, and thus, the role that ADP has in shaping the adaptive immune response.

4.1.2 Effect of ADP on CD4\(^+\) T Cell Responses

In our model, ADP did not affect DC-mediated T cell activation, indicating that the inhibited proliferation I initially observed in co-cultures may be due to specific effects on T cells. To elucidate the role of ADP in T cell responses, T cells were activated in vitro with \(\alpha\)-CD3, \(\alpha\)-CD28 coated microbeads to eliminate any effects of ADP on the APC. These beads are able to provide signal 1 (ligation of CD3 and TCR) and signal 2 (CD28 binding), which are required for effective T cell activation (Liwski et al., 2006, Wells, 2009). When activated with microbeads in the presence of ADP, proliferation of CD4\(^+\) T cells was inhibited in a dose-dependent manner, with significant inhibition occurring in the presence of 50 \(\mu\)M ADP. Interestingly, the effect of ADP\(\beta\)S on T cell responses was more potent, with all tested concentrations causing a significant reduction in proliferation. ADP\(\beta\)S may be a stronger inhibitor of T cell proliferation because it is not being degraded and thus remains in culture longer. It is noteworthy here that the inhibitory effect of ADP and ADP\(\beta\)S appear to be more potent on T cells in the absence of DCs, suggesting that perhaps ADP and ADP\(\beta\)S are having an additional effect on the DCs that I was unable to observe in my experimental model. Importantly, the reduced CD4\(^+\) T cell proliferation observed was not due to ADP- or ADP\(\beta\)S-mediated toxicity.

Following receipt of co-stimulatory signal 2, T cells produce the growth factor IL-2, and expression of the high affinity IL-2 receptor (CD25) is promoted (Smith, 1980). Together these molecules interact in an autocrine manner to promote clonal expansion of
T cells (Smith, 1980, Malek and Bayer, 2004). IL-2 production by T cells was significantly reduced by the presence of ADP or ADPβS. I attempted to rescue CD4⁺ T cell proliferation by adding an excess of exogenous IL-2 to T cell cultures during activation. Interestingly, in the presence of ADP or ADPβS, exogenous IL-2 was unable to significantly increase T cell proliferation. This may be explained by the reduced surface expression of CD25 on CD4⁺ T cells activated in the presence of ADP or ADPβS.

Since activated T cells produce a number of cytokines in addition to IL-2 to promote their own expansion (Geginat et al., 2003), I tried adding supernatant from previously activated T cells to CD4⁺ T cell cultures activated in the presence of ADP or ADPβS. Again, I was unable to restore T cell proliferation, indicating that ADP may be inhibiting signaling events involved in the activation of T cells.

Full activation of a T cell necessitates signals from TCR to initiate signaling, costimulation (e.g. through CD28) to augment TCR-signals, and cytokine help to promote clonal expansion of effector cells (Smith-Garvin et al., 2009). Signaling from the TCR and CD28 culminate in the activation of ERK1 and ERK2 through the MAPK pathway, as well as activation of Akt downstream of PI3K (Meloche and Pouyssegur, 2007, Kane and Weiss, 2003). ERK1/2 and Akt mediate activation of important T cell transcription factors, including NF-κB, AP-1 and NFAT (Smith-Garvin et al., 2009). When CD4⁺ T cells were activated by coated microbeads in the presence of ADP or ADPβS, I found that phosphorylation of ERK1 appeared to be decreased 5 min after activation. Similarly, Akt phosphorylation also seemed to be decreased 60 min following CD4⁺ T cell activation in the presence of ADP or ADPβS.
Naïve CD4\(^+\) T cells were found to express mRNA for the ADP receptor P2Y\(_1\), which elicits cellular responses by coupling to the G\(\alpha_q\) protein (Leon et al., 1997). Interestingly, G\(\alpha_q\) signaling inhibits activation of PI3K, and downstream Akt (Harris et al., 2006), which may be responsible for the apparent decrease in Akt phosphorylation observed. Signaling by G\(\alpha_q\) is also responsible for activation of the MAPK pathways (including ERK1/2) through activation of PLC\(\beta\) and DAG generation (von-Kgelgen and Harden, 2011). The apparent decrease in ERK1 phosphorylation observed is not consistent with G\(\alpha_q\) signaling, suggesting that another pathway mediated through ADP ligation of P2Y\(_{12}\) or P2Y\(_{13}\) may be involved. Although naïve CD4\(^+\) T cells were not found to express mRNA for P2Y\(_{12}\) or P2Y\(_{13}\), it is possible that their transcription becomes up-regulated following T cell activation. These receptors are coupled to G\(\alpha_i\) (Foster et al., 2001, Zhang et al., 2002), which has been associated with decreased ERK1/2 activity. Decreased activation of ERK1/2 can be lead to less intracellular Ca\(^{2+}\) (Goldsmith and Dhanasekaran, 2007), suggesting that subsequent NFAT activity and IL-2 production may also be hindered. This signaling may be responsible for the inhibited IL-2 secretion that I observed, however further studies into NFAT activation would have to be completed to validate this.

ADP may also affect transcription factors involved in clonal expansion of CD4\(^+\) T cells. For example, STAT5 activation is impaired by adenosine, resulting in reduced IL-2 secretion (Zhang et al., 2004), and may also be affected by ADP in the current study. Importantly, transcriptional activity of NF-\(\kappa\)B, NFAT and AP-1 should also be investigated, as these molecules are important in naïve T cell activation (Smith-Garvin et al., 2009).
Together my results implicate ADP as an immunosuppressive mediator, with the ability to strongly inhibit CD4⁺ T cell activation and proliferation. I also demonstrated that migration of naïve CD4⁺ T cells to ADP or ADPβS was significantly inhibited. Interestingly, Idzko et al. (2002) showed that the presence of ADP lead to enhanced DC chemotaxis. These contradictory findings may be cell type dependent, or may be due to specific ADP receptor ligation, as P2Y₁ and P2Y₁₂/₁₃ are coupled to different Gα signaling pathways. Signaling through Gαi, which is initiated from P2Y₁₂/₁₃ ligation, or from CCL19 interaction with CCR7, can lead to enhanced migration (Riol-Blanco et al., 2005), while Gαq signaling has been suggested to decrease migration (Molon et al., 2005). Future studies are required to confirm which receptor is responsible for the observed ADP effect on DCs and T cells.

During cancer progression, metastatic foci can be established in lymph nodes. ATP generated from these tumors (Aymeric et al., 2010) can be degraded to ADP. Therefore, if ADP was present in draining lymph nodes during cancer metastasis, recruitment of naïve T cells and their subsequent activation would be quite impaired, leading to decreased immunosurveillance. It would be interesting to investigate if migration of activated T cells to ADP or ADPβS was also impaired, as this could have implications in the adaptive response where effector T cells are recruited to sites of injury.

### 4.1.3 Effect of ADP on CD8⁺ T Cell Responses

Since naïve CD8⁺ T cells were found to also express mRNA for the P2Y₁ ADP receptor, I thought it would be interesting to see if the effect of ADP or ADPβS on this cell population was similar to that on CD4⁺ T cells. When exposed to the nucleotides
during activation with α-CD3 and α-CD28 coated microbeads, proliferation of CD8^+ T cells was not significantly decreased. Interestingly, CD25 expression was significantly inhibited on CD8^+ T cells following stimulation. However, as CD8^+ T cells are less dependent than CD4^+ T cells on IL-2 autocrine signaling (D'Souza and Lefrancois, 2003), the observed decrease in CD25 does not necessarily have to correlate to decreased CD8^+ T cell activation. Additionally, the inhibitory effect of ADP may depend on receptor expression, as CD8^+ T cells appeared to have less P2Y1 mRNA than CD4^+ T cells.

IFN-γ is an important cytokine for effector CD8^+ T cell responses, which includes anti-viral immunity and cytolytic activity (Sercan et al., 2006). In the presence of ADP or ADPβS, IFN-γ secretion by CD8^+ T cells was decreased by 75-80%. Surprisingly, CTLs generated in the presence of ADP or ADPβS did not have altered cytotoxic activity. In the model that I used, ADP was only present during CTL generation. It would be interesting to see how CD8^+ T cell killing activity was affected if ADP was present during targeted lysis, as this would have implications during viral clearance following tissue injury. Also, to fully elucidate the role of ADP in CD8^+ T cell responses, it will be important to investigate the production of lytic mediators, including cytolytic granules, and Fas ligand (Bossi and Griffiths, 2005, Fan and Zhang, 2005) in the presence of ADP or ADPβS.

### 4.1.4 Effect of ADP on B Cells

B cells undergo rapid proliferation following encounter with a danger signal, enhancing DC-mediated T cell responses and promoting memory immune responses.
(Cerutti et al., 2011). Although non-activated B cells appear to express low levels of P2Y13 mRNA, expression of ADP receptors in activated cells, where transcription may be increased, was not investigated. In a pilot study, I observed that 50 µM ADPβS, but not ADP, caused a significant increase in B cell proliferation. The more potent effect of ADPβS on B cells is likely due to its increased stability in vitro. However, this result suggests that ADP may have pro-inflammatory activity, similar to what Ben Addi et al. (2010) observed with DC antigen presentation and others have witnessed in disease models (Abele, 2009, Hagiwara et al., 2011). Important aspects of B cell function, including antibody production, antigen presentation and cytokine production (Nimmerjahn and Ravetch, 2010, Lund, 2008), may also be affected by ADP, and would be the focus of future studies.

4.2 FUTURE DIRECTIONS

Collectively, these results suggest that the effect of ADP is cell type-dependent, and that more investigation is required to fully appreciate the role of ADP in regulating the adaptive immune response. While I was unable to observe ADP-induced differences in DC activity in the current study, others have shown that ADP increases antigen uptake by DCs (Ben Addi et al., 2010), which could enhance antigen presentation. Interestingly, I demonstrated that ADPβS could increase B cell proliferation in a pilot study, which may further suggest that ADP has a pro-inflammatory role in initiating the immune response. Conversely, I have shown that ADP can be potently immunosuppressive, significantly inhibiting the proliferative capacity of CD4+ T cells, and preventing IL-2 mediated T cell expansion. Additionally, while CD8+ T cell killing activity was unaffected, ADP
significantly impaired CD25 expression and IFN-γ secretion, indicating that other CD8⁺ T cell activities may be affected. The suppressive properties of ADP that I have observed in CD4⁺ T cell responses are quite similar to those of adenosine (Erdmann et al., 2005). It is possible that adenosine generation by ectonucleotidases may contribute to some observations, although it is unlikely in assays performed with ADPβS. To confirm that the observed CD4⁺ T cell suppression is ADP-mediated and not due to adenosine, non-selective P2 receptor inhibition by suramin could be used (Ralevic and Burnstock, 1998). If a decrease in proliferation is still observed, the inhibition may be adenosine-mediated.

The cytokine milieu during effector T cell activation largely shapes the resulting T cell response. As mentioned, ADP has been shown to decrease IL-12 production by DCs (Marteau et al., 2004), which suggests polarization away from type 1 responses. However, the secretion of other cytokines by DCs has not been examined. It would be important in future studies to investigate not only the cytokines released by DCs in the presence of ADP, but also those released from activated T cells. In the current study, production of the type 1 cytokines IL-2 and IFN-γ was decreased in the presence of ADP. Secretion of type 2 (IL-4, IL-5, IL-13), Th17 (IL-17, IL-23) and Treg (TGF-β, IL-10, IL-35) cytokines following activation in the presence of ADP has not been investigated.

ADP can bind to three receptors, which trigger cellular responses by coupling to different Gα-proteins (P2Y₁ is Gαq coupled, while P2Y₁₂/₁₃ are Gαi coupled) (Burnstock and Knight, 2004). Interestingly, a recently published study showed that, with respect to insulin production by β-islet cells in the pancreas, ADP receptors could have opposing functions in that ADP-mediated signaling through P2Y₁ lead to increased insulin production, while P2Y₁₃ ligation resulted in decreased insulin (Amisten et al., 2010). It is
possible, therefore, that cellular responses to ADP within the immune system are dictated by receptor expression. DCs were found to express all known receptors for ADP, but which signaling pathway dominates and is responsible for the observed phenotype is unknown. In naïve CD4⁺ and CD8⁺ T cells, only P2Y₁ receptor expression was detected, suggesting that suppressed responses may be a result of Gαᵣ signaling. A number of pharmacologic agents are commercially available that selectively and non-selectively target ADP receptors and can be used for *in vitro* studies. For example, P2Y₁ can be potently inhibited by N⁶-methyl-2’-deoxyadenosine 3’, 5’-bisphosphate (MRS2179) (Burnstock and Knight, 2004). P2Y₁₂ and P2Y₁₃ are Gαᵢ-coupled, so pertussis toxin could be used to non-specifically target Gαᵢ-mediated responses. Alternatively, P2Y₁₂ can be reversibly and selectively inhibited by AR-C69931MX (also known as Cangrelor, which is currently undergoing trials for clinical use) or AR-C67085, which can also antagonize P2Y₁₃ responses at high doses (Erlinge, 2011).

ADP signals through both P2Y₁ and P2Y₁₂ in the process of platelet activation. First, Ca²⁺ mobilization and platelet shape change is induced following P2Y₁ ligation (Jin et al., 1998), and then platelet aggregation is induced following P2Y₁₂-mediated release of ADP granules from platelets (Dorsam and Kunapuli, 2004). Since P2Y₁ signaling affects platelet shape, and Idzko et al. (2002) have previously shown that ADP can enhance chemotactic ability by actin polymerization in DCs, it is possible that ADP affects also cytoskeletal rearrangements of DCs and/or T cells during immunological synapse formation. Additionally, ADP may also affect the clustering of TCR and co-stimulatory molecules required for T cell activation. In future studies, fluorescent
microscopy could be used to view cytoskeletal changes, and aggregation of the TCR and co-stimulatory molecules at the synapse between T cells and APCs.

Eventually, it will be important to bring these studies to in vivo models. To my knowledge, this is the first demonstration of direct regulation of CD4+ and CD8+ T cell responses by ADP. These findings are of particular relevance to a number of physiological systems, including wound healing and immune evasion by cancer cells, in which effector T cell responses are required for effective clearance of pathogens or and neoplastic cells. Knockout mice are commercially available for all three ADP receptors, which will facilitate in vivo studies. Furthermore, antagonism of the P2Y_{12} ADP receptor with pro-drug clopidogrel has been used previously in animal models of transplant arteriosclerosis (Abele, 2009) and sepsis (Hagiwara et al., 2011). MRS2179 antagonism of P2Y_{1} has also been used in animal models (Erlinge, 2011), but as of yet, there are no clinical agents that target P2Y_{1}. However, since clopidogrel and the newer generation drug prasugrel are used clinically to antagonize P2Y_{12}-mediated platelet activation with limited adverse effects (Erlinge, 2011), translation from animal models to human trials is possible.

4.3 CONCLUSIONS

CD4+ T cell activation and proliferation were significantly inhibited in the presence of ADP, which was associated with reduced CD25 expression and IL-2 secretion in vitro. ADP also reduced IFN-γ secretion by CD8+ T cells, indicating that ADP may also suppress certain CTL responses. Interestingly, ADP appears to be pro-inflammatory in APCs, causing increased B cell proliferation. The effect of ADP may be regulated by
receptor signaling, therefore implicating ADP in ‘fine tuning’ of the adaptive immune response.
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