Regulation of the Pro-Inflammatory Cytokine TNF-α by Adenosine Receptors in Monocytes and Macrophages

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Abbreviations

AB-MECA: N- (4-Amino-3-iodobenzyl)-5´-N-methylcarbamoyladenosine 1-[6-[[(4-Amino-3-iodophenyl)methyl]amino]-9H-purin-9-yl]-1-deoxy-N-methyl-β-D-ribofuranuronamide 3-Iodo-4-aminobenzyl-5´-N-methylcarboxamidoadenosine

ATF-2: Activating transcription factor 2

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

BBB: Blood brain barrier

cAMP: Cyclic adenosine monophosphate

CCPA: 2-Chloro-N^6-cyclopentyladenosine

CGS 21680: 2-p-(2-Carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride

CNS: Central nervous system

DPCPX: 8-Cyclopentyl-1,3-dipropylxanthine

EAE: Experimental autoimmune encephalomyelitis

4EBP-1: Eukaryotic initiation factor 4E binding protein 1

eIF-4E: Eukaryotic initiation factor 4E

ERK: Extracellular signal-regulated protein kinase

H89: N-[2-((*p*-Bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide

IB-MECA: 1-Deoxy-1-[6-[((3-Iodophenyl)methyl)amino]-9H-purin-9-yl]-N-methyl-b-D-ribofuranuronamide N^6-(3-Iodobenzyl)adenosine-5´-N-methyluronamide

IFN-γ: Interferon γ

IL: Interleukin

JNK: cJun N terminal kinase

MAPK: Mitogen-activated protein kinase

MHC: Major histocompatibility complex

MDL 12330A: *cis*-N-(2-Phenylcyclopentyl)azacyclotridec-1-en-2-amine, HCl

MRS 1220: 9-Chloro-2-(2-furanyl)-5-((phenylacetyl)amino)-[1,2,4]triazolo[1,5-c]quinazoline

MS: Multiple sclerosis

NFAT: Nuclear factor of activated T cells

NF-κB: Nuclear factor κB

PD 98059: 2′-Amino-3′-methoxyflavone

PKA: Protein kinase A

PKC: Protein kinase C

PP1: Protein phosphatase 1

PP2A: Protein phosphatase 2A

8-PT: 8-phenyltheophylline

R-PIA: (R)-N^6-(1-Methyl-2-phenylethyl)adenosine

SB 202190: 4-(4-Fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole

SP 600125: Anthra[1,9-*cd*]pyrazol-6(2*H*)-one 1,9-pyrazoloanthrone

TH1/TH2: T helper 1/T helper 2

TNF-α: Tumour necrosis factor α

U73122: 1-[6-((17b-3-Methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione

ZM 241385: 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol

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Abstract

Activation and control of immune and inflammatory responses provides protection to the host while minimizing development of disease and damage to healthy tissue. Endogenous substances can modulate the initiation and duration of immune and inflammatory responses. Production of the purine nucleoside adenosine is increased during inflammation and adenosine has been proposed to act as a "sensor" of damage and a controller of immune responses. Accordingly, we designed experiments to identify cell signaling pathways that mediate immune-modulatory effects of adenosine, focusing on control of the pro-inflammatory cytokine TNF-α. Cytokines are essential for communication between immune cells and their expression can control the activation, type, and duration of developing immune responses. TNF-α in particular controls activation of phagocytes, T lymphocytes, and contributes to leukocyte migration. As a result, adenosine-mediated control of TNF-α can influence both innate and adaptive immune responses. Using U937 cells as a model of monocytes and macrophages, the primary producers of TNF-α, and phorbol esters as an inflammatory stimulus, we showed that activation of adenosine receptors inhibited TNF-α protein production. CGS 21680, an agonist of adenosine A_{2A} receptors, was the most potent and efficacious adenosine receptor agonist tested and inhibited TNF-α production post-transcriptionally by decreasing stability and half-life of TNF-α mRNA. CGS 21680 decreased phospho-p38 protein levels, suggesting that A_{2A} receptor activation decreased activity of the p38 signaling pathway which is normally involved in stabilizing mRNAs encoding transiently

expressed proteins such as TNF-α. The anti-TNF-α effects of CGS 21680 required protein phosphatase activity and activation of A_{2A} receptors increased cytosolic serine/threonine phosphatase activity. By increasing phosphatase activity, A_{2A} receptor activation may efficiently control signaling pathways involved in regulating TNF-α production including p38. Adenosine A_{2A} receptors may also control p38 signaling by decreasing expression of GADD45, a protein involved in upstream control of p38 activity. In addition to controlling TNF-α production, adenosine A_{2A} receptors may modulate monocyte and macrophage activity by decreasing cell adhesion and decreasing expression of cell-surface adhesion molecules. By modulating recruitment of monocytes and macrophages to an inflammatory site and decreasing cytokine production by those cells, adenosine A_{2A} receptors can efficiently control the participation of macrophages in a developing immune response. Here, we propose a model whereby adenosine acts during the initiation of an inflammatory response, first through control of cytokine production to prevent tissue damage until the appropriate required immune response is determined and initiated, and second through mast cells to relay information about the degree of inflammation and tissue damage. Adenosine then modulates innate immunity by controlling neutrophil and macrophage function and adaptive immunity by controlling cytokine production and T lymphocyte proliferation and activation. Adenosine A_{2A} receptor activation modulates phosphorylation-dependent signaling pathways to efficiently control TNF-α and as such, may provide a novel therapeutic target for intervention in inflammatory disorders associated with superfluous cytokine production.

General Introduction

1.1 Immune and Inflammatory Responses[1]

Immune and inflammatory responses are designed to help protect the body from infection and to assist in the repair of tissue damage. Inflammation and immune responses are distinct and different reactions. Inflammation is activated by physical tissue injury and is designed to promote tissue repair with classical symptoms that include edema, heat, and redness. On the other hand, the immune system is a complex network of cells and proteins whose physiological role is to destroy pathogens and eliminate infection from the body. Components or products of infectious pathogens like bacteria and viruses activate immune responses. Although they can exist separately, immune and inflammatory responses both share common cellular (eg. phagocytes) and soluble mediators (eg. cytokines) and as a result can also work together to ultimately protect and repair the body. This introduction is designed to give an overview of inflammation and immune responses detailing the cellular mechanisms involved in each and to introduce the adenosine system. Specific details outlining the role of adenosine in immune and inflammatory responses will be given in introductions to specific Chapters.

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[1] Section 1.1 – 1.7 of the introduction are written largely without references as they cover basic information about the immune system and inflammatory responses. References used to prepare this section include the following introductory immunology texts:

Abbas et al., Cellular and Molecular Immunology Fourth Edition, W.B. Saunders Company, Toronto, ON, 2000.

Janeway et al., Immunobiology: The Immune System in Health and Disease, Garland Publishing, New York, NY, 2001.

Thomson, Angus, The Cytokine Handbook Third Edition, Academic Press, San Diego, CA, 1998.

1.2 Innate and Adaptive Immunity

Defense against infectious agents occurs through innate and adaptive immunity. Innate immunity responds to conserved, broadly expressed antigens common to many infectious agents like lipopolysaccharide. As a result, innate immune responses have broad specificity, they are fast and occur early during infection, they do not require pre-sensitization with an infectious agent, they exhibit no memory towards the activating antigen, and in most cases the intensity of the response is the same upon subsequent exposure to the same pathogen. The principal factors mediating innate immunity are soluble mediators (complement, acute phase proteins, cytokines) and cellular mediators (phagocytic cells including neutrophils and macrophages, natural killer cells, mast cells, $\gamma\delta$ T cells, CD5+ B cells, basophils, eosinophils, and dendritic cells).

Whereas broadly expressed antigens activate innate immune responses, highly specific antigens activate adaptive immune responses. Adaptive immunity occurs later during an infection and is mediated by T and B lymphocytes. Adaptive immune responses depend on activation and clonal expansion of T and B lymphocytes, resulting in a response that is slower than innate responses. In addition, adaptive immune responses require pre-sensitization with the infectious antigen, are highly specific and exhibit both T and B lymphocyte memory.

Although innate and adaptive immune responses employ different mechanisms of defense and involve different cell types, communication and interactions

between these two different responses can occur and may be required for an effective immune response against a particular pathogen. For example, macrophages participate in innate immune responses by phagocytosing and destroying pathogens or cells infected with pathogen. However, during adaptive immune responses, macrophages can phagocytose, process, and present antigen to naive T lymphocytes, resulting in their activation. In addition, macrophages are activated by TH1 cells during an adaptive immune response and are the ultimate effector cell of adaptive immunity. Consequently, immune cells can perform a variety of functions during different phases of immune responses. Therefore, natural, experimental or pharmaceutical means of interfering with the function of one type of immune cell can have numerous downstream effects on other cellular mediators of immune responses.

When a microbe passes epithelial barriers, which provide initial protection from infection, it initiates the recognition, activation and effector mechanisms of innate immunity. These mechanisms are carried out by the cellular mediators of innate immunity, which respond to pathogens by signaling their destruction and removal. Phagocytes (macrophages and neutrophils) are designed to recognize, phagocytose and destroy both extracellular pathogens and cells infected with intracellular pathogens. The recognition phase (phagocyte activation) occurs through highly conserved and broadly expressed components of pathogens like lipopolysaccharide, a component of gram-negative bacterial cells walls. Pathogens and infected cells can also become coated with antibodies or

complement, a process that signals activation of and destruction by phagocytes (opsonization). Host cells that become infected with an intracellular pathogen (bacteria or virus) can express antigen from that pathogen via MHCI. The MHCI expressed antigen activates CD8+ cytotoxic T lymphocytes that then respond by lysing the infected cell. Some bacteria and viruses have evolved to decrease expression of MHCI once they infect a host cell in an effort to evade destruction by the immune system. Natural killer cells serve to destroy host cells infected with intracellular pathogens only in the absence of MHCI, a mechanism which can destroy bacteria or virus that have evaded MHCI dependent immune responses. Innate and adaptive immune responses to pathogens are summarized in Figure 1.

Soluble mediators like complement and cytokines play an integral role in the development of innate immune responses (Figure 1). Complement proteins form a cascade of activated proteins in the plasma that can destroy bacteria or cells by forming a membrane attack complex that punches holes in bacterial or cell membranes. Complement proteins also contribute to innate immunity by coating bacteria and cells infected by intracellular pathogens, as described earlier, thus signaling them for destruction by phagocytes. Finally, cytokines and chemokines contribute to innate immunity by activating phagocytes, by attracting other white blood cells to sites of infection and injury, and by assisting in death of cells infected with intracellular pathogens.

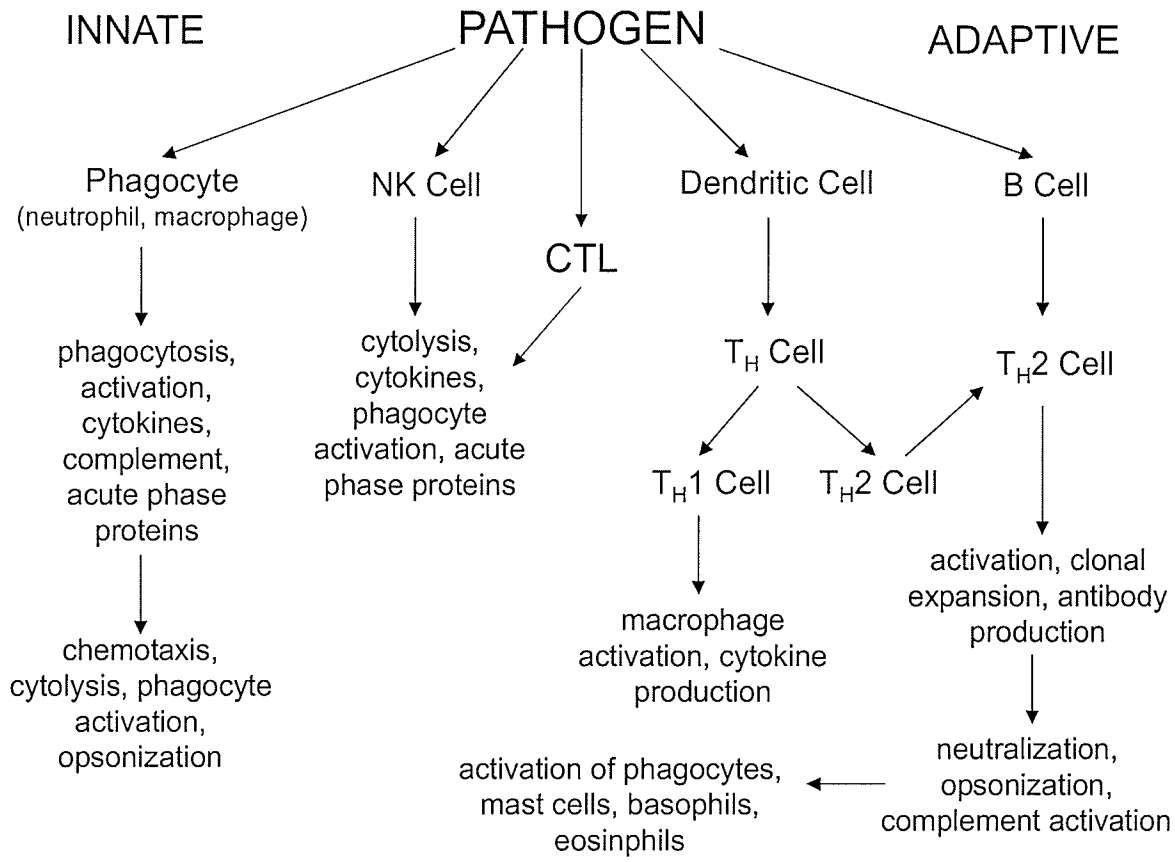

Figure 1: Summary of Innate and Adaptive Immune Responses to Infection with Pathogen. NK cells – natural killer cells, CTL – cytotoxic T lymphocytes.

Like innate immune responses, adaptive immunity involves recognition, activation and effector phases (Figure 1). The recognition phase of adaptive immunity occurs early during an infection and involves recognition of foreign antigens by antigen presenting cells. Dendritic cells, the most important antigen presenting cells, phagocytose pathogens, process pathogens, and express processed antigen via MHCII to naïve T cells. T cells interact with antigen presenting cells in lymph nodes such that when a T cell expressing a receptor that recognizes a particular antigen expressed by MHCII interacts with its

corresponding antigen presenting cell, that T cell becomes activated and undergoes proliferation, differentiation, and clonal expansion. Depending on the presense of different cytokines during T cell differentiation, these CD4+ cells can become either TH1 or TH2 cells. TH1 cells predominantly act to assist the destruction of pathogens by macrophages and are produced in response to cytokines like IL-12 and IFN-γ. TH2 cells assist in the activation of B cells, resulting in proliferation, clonal expansion, and differentiation of B cells to plasma cells, which then produce and secrete antibody. During the recognition phase of adaptive immunity, naïve/intact antigen can also be recognized and phagocytosed by B cells that process and present antigen via MHCII to TH2 cells. This interaction leads to the activation and differentiation of B cells. Activation of a TH1 response results in cell-mediated immunity, whereas activation of B cells and a TH2 response results in humoral immunity.

Adaptive immunity depends on antigen recognition by T and B lymphocytes. T lymphocytes can express either CD4 or CD8. CD4+ T cells are activated by MCHII presented antigen and differentiate into either TH1 or TH2 cells. CD8+ cells, on the other hand, are activated by MHCI presented antigen and release toxic granules upon activation which lyse the target cell expressing the antigen.

Activation of both T and B lymphocytes is dependent upon initial recognition of antigen by either T cell receptors or B cell receptors, however activation of these cells is dependent on the presence of secondary signals. For example, T cells

express CD28 that interacts with B7 on dendritic cells thereby providing the co-stimulatory signal required for activation. Activation of B cells depends on interaction of the B cell presenting antigen via MHCII with TH2 cells. TH2 cells provide cytokines (IL-4 and IL-5) and CD40:CD40 ligand interactions which signal activation of B cells and assist in antibody production.

Once antigen is eliminated by the effector mechanisms of innate and adaptive immunity, effector cells either undergo cell death, which terminates the immune response, or become quiescent memory lymphocytes, which are armed to launch a specific response upon re-exposure to the activating pathogen.

1.3 Inflammatory Responses

Inflammatory responses are activated by tissue damage and are defined as a reaction of the microcirculation resulting in edema, redness, heat and pain. Inflammatory reactions are either acute or chronic, generally defined by the cell types mediating the response. Acute inflammatory reactions involve neutrophils, mast cells, and platelets whereas chronic inflammatory reactions involve macrophages, lymphocytes, and plasma cells. Both reactions can result in similar physiological responses, namely increased vascular permeability, vasodilation, chemotaxis, margination, adherence, and emigration of lymphocytes and increased flow of lymph. Because the cell types and physiological responses overlap between immune and inflammatory responses, they can co-exist in an effort to heal an injured or infected tissue. The actions of

cells involved both during inflammation and immune responses are modulated by cytokines and other soluble mediators like chemokines that participate by assisting in cellular activation, recruitment and migration across endothelial barriers.

1.4 Role of Monocytes and Macrophages in Immune and Inflammatory Responses

Macrophages originate in the bone marrow, enter the peripheral blood as monocytes, and differentiate into macrophages in tissues. Monocyte differentiation is triggered by interactions with endothelial cells via adhesion molecules and by subsequent migration into tissues. Differentiation is assisted by cytokines that alter adhesion molecule expression and by chemokines that recruit monocytes. Macrophages play a key role both in innate and adaptive immunity. During innate immune responses, macrophages phagocytose and kill pathogens and cells infected by pathogens. Macrophages can either recognize the pathogen or infected cell directly or indirectly through antibody or complement which coat the pathogen or cell and signal it for destruction by phagocytes. In addition, phagocytes are also activated by TH1 cells and serve as the ultimate effector of cell-mediated immunity during an adaptive immune response. Tissue resident macrophages also take up and process antigen, express processed antigen via MHCII and can participate in adaptive immunity by activating CD4+ TH cells. Finally, activated macrophages can propagate and

direct immune responses by producing and releasing cytokines whose functions will be discussed in Section 1.5.

The presence of macrophages at an inflammatory site is a hallmark of chronic inflammation where they perform similar functions as in immune responses. Macrophages aid in healing by assisting in removal of dead or destroyed tissue. Macrophages produce and secrete soluble mediators like cytokines and chemokines, which as in immune responses direct the activation and recruitment of accessory immune cells. Finally, macrophages contribute to effector phases of lymphocyte activation as well as to lymphocyte activation itself, mechanisms that aid in prevention of infection at sites of inflammation and tissue injury.

1.5 Cytokines and Immune Responses

An important function of macrophages is the production of cytokines, however immune and non-immune cells can also produce these proteins. Like macrophages, cytokines participate in innate and adaptive immunity as well as in inflammation. Cytokines contribute to growth, differentiation and activation of lymphocytes (IL-2, IL-4, IL-5, IL-10, IL-12), activation of macrophages (TNF-α, IFN-γ), chemotaxis (IL-8), cell death (TNF-α) and inflammation (IL-1). Generally, cells do not contain stores of cytokines ready to be released upon stimulation. As a result, cytokine production is dependent on gene transcription and protein synthesis. Cytokines can act on a variety of cell types by interacting with different receptors like G protein coupled receptors and tyrosine kinase

receptors, which accounts for the diversity of their functions. Cytokines also exhibit redundancy of function meaning that different cytokines can have similar functions and may compensate or take over for each other. For example, TNF-α and IL-1 have similar functional profiles and as a result, IL-1 can help replace the functions of TNF-α if its activity or production is blocked. Cytokines can also negatively affect the actions of other cytokines through antagonism, or positively affect cytokine actions in additive or synergistic ways. For example, IFN-γ can exert such a positive effect by increasing the production of TNF-α by activated macrophages, and IL-10 can negatively affect TNF-α by blocking its production. The functions of cytokines are local (autocrine or paracrine) and systemic (endocrine). Because the effects of cytokines are mediated through interactions with specific receptors, the responsiveness of a cell to a particular cytokine can be influenced by the expression of cytokine receptors on that cell. Immune responses can be regulated then both by influencing cytokine production as well as cytokine receptor expression. Cytokine-cytokine receptor interactions generally result in activation of gene expression, however cytokine receptor activation can also result in cellular proliferation, migration (chemokines), and cell death (TNF-α).

The principal cytokines that mediate innate immunity include TNF-α, IL-1, IL-12, and IFN-γ. These cytokines are produced by most immune cells, particularly macrophages, and contribute to activation of phagocytes and differentiation of TH1 cells. The principal mediators of adaptive immunity include IL-2, IL-4, IL-5

and IFN-γ. These cytokines are produced and released from antigen-stimulated T lymphocytes and other immune cells like macrophages. They stimulate proliferation, differentiation and activation of both TH2 and B cells, resulting in generation of plasma cells, which produce and secrete antibody.

1.6 TNF-α Production and Signaling in Immune Responses

TNF-α is primarily involved in and is a key mediator of innate immunity. TNF-α is produced by a variety of cell types including activated T lymphocytes, natural killer cells, and mast cells but predominantly by monocytes and macrophages. TNF-α production is initiated by several stimuli most notably by gram-negative bacteria and lipopolysaccharide and it plays an integral role in cell-mediated immunity by both activating effector cells (macrophages and neutrophils) and signaling cell death (of cells infected by intracellular pathogens when released by CD8+ cytotoxic T lymphocytes) (Tracey, 2002). Monocyte and macrophage production of TNF-α results from gene transcription and translation; this involves activation of several cell-signaling pathways and production of a 26 kD precursor TNF-α that resides in the plasma membrane. The large carboxy terminus is cleaved at the cell membrane by TNF-α converting enzyme and is released from the cell as a 17 kD protein (Black et al., 1997; Mohan et al., 2002). Both soluble and membrane-bound TNF-α exists as a homotrimer and can interact with two TNF-α receptors, TNFRI (p55) and TNFRII (p75). Although the majority of cells types express both TNF-α receptors, most of the biological effects of TNF-α are mediated through TNFRI, which involves activation of caspases and apoptosis as

well as activation of transcription factors. Activation of TNFRII involves recruitment of TNFR-associated factors (TRAFs) to the receptor and activation of transcription factors like AP-1 and NF-κB (Cohen, 2002; Kassiotis and Kollias, 2001; MacEwan, 2002).

1.7 TNF-α Functions

The physiological functions of TNF-α within the immune system are numerous and include recruitment and activation of mononuclear phagocytes to sites of infection, increased production of adhesion molecules in vascular endothelial cells, increased chemotaxis and recruitment of leukocytes, and apoptosis. By activating adhesion and recruiting immune cells to an infection site, TNF-α serves a protective role as a positive mediator of acute inflammatory responses and innate immunity. However, TNF-α can also contribute to pathological conditions when its production becomes excessive, a situation which can occur during severe infections like sepsis.

When TNF-α levels become very high (plasma concentrations $\geq 10^{-7}$ M), TNF-α can enter the blood stream and function in an endocrine manner. In the hypothalamus, TNF-α can induce prostaglandin synthesis resulting in fever. In the liver, TNF-α stimulates serum protein production as part of acute phase responses. TNF-α can lead to metabolic conditions characterized by wasting of muscle and fat due largely to cytokine-induced appetite suppression and the inability of the liver to replace glucose used by muscle. TNF-α acts on the heart

and cardiovascular system to decrease contractility and smooth muscle tone, resulting in low blood pressure and eventually septic shock. Vascular thrombosis can also occur as a result of coagulation factor activation and inhibition of anti-coagulant factors. Incidentally, thrombus activation results in tumor necrosis, the property TNF-α was discovered for. In general, normal inflammatory and immune functions of TNF-α occur when plasma concentrations are less than 10^{-9} M. Moderate levels of TNF-α, between 10^{-9} and 10^{-7} M act on the brain and liver. High levels of TNF-α (when plasma concentrations are higher than 10^{-7} M) cause septic shock resulting from actions on the heart, vasculature, and induction of hypoglycemia.

1.8 Inflammatory Disorders

Although our immune systems are designed to protect us against infection and disease, immune-related diseases arise when regulation of immune responses malfunctions. Autoimmune disorders (Type 1 diabetes, multiple sclerosis, Lupus) develop when the immune system attacks the host and causes tissue damage and disease (Benoist and Mathis, 2002). Also, we can see in the case of TNF-α that if cytokine levels are not adequately controlled, serious pathological consequences can occur (Cohen, 2002; Kassiotis and Kollias, 2001). As a result, inactivation and control of immune responses is just as important as activation of immune responses for defense against infection and disease. When an immune response is not adequately controlled or shut off, diseases like Crohn's disease and rheumatoid arthritis can develop. In addition, we now know

that many disorders and diseases such as ischemic and hemorrhagic stroke, Alzheimer's disease and HIV-associated dementia have immune components (Nathan, 2002). To understand the progression and development of these disorders and to develop novel therapeutic targets for them, it is essential to understand how immune responses are normally regulated, controlled and turned off. The focus of this work is towards understanding how the adenosine system influences and controls immune and inflammatory responses.

1.9 Adenosine Production and Control of Adenosine Levels

The adenine nucleoside adenosine is formed from metabolism and breakdown of adenosine triphosphate (ATP) during energy requiring processes, and adenosine is formed and released from cells that are metabolically active and/or stressed. Different degrees of energy expenditure contribute to different concentrations of adenosine present both intra- and extra-cellularly. Levels of adenosine are controlled by production, metabolism and transport. 5'-nucleotidase is an enzyme that directly increases adenosine production from adenosine monophosphate (AMP). Adenosine kinase and adenosine deaminase are enzymes primarily responsible for adenosine metabolism and convert adenosine to AMP and inosine, respectively. Adenosine levels are also controlled by transport into and out of a cell via facilitated diffusion, fascilitative or equilibrative transporters, and sodium-dependent concentrative transporters. A summary of adenosine production, metabolism, and transport is shown schematically in Figure 2.

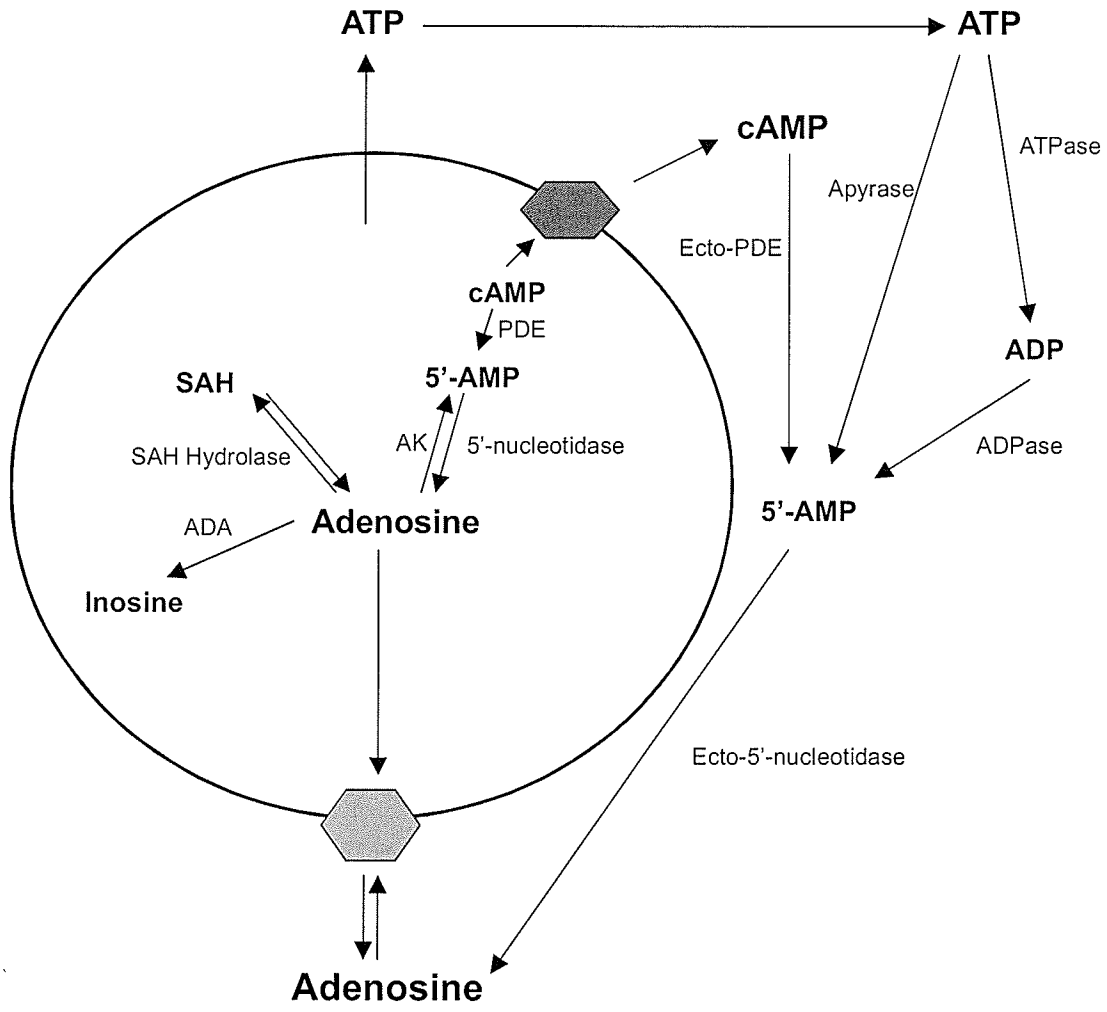

Figure 2: Adenosine Production, Metabolism, and Transport. Abbreviations: PDE – phosphodiesterase, ADA – adenosine deaminase, SAH – S-adenosylhomocysteine, AK – adenosine kinase, cAMP – cyclic adenosine monophosphate, AMP – adenosine monophosphate, ADP – adenosine diphosphate. Green – nucleoside transporter, blue – cAMP transporter

Adenosine levels are increased during hypoxia, ischemia, hypoglycemia, seizures, free radical production, and electrical and synaptic stimulation (Dunwiddie and Masino, 2001). Experimentally, increased endogenous

adenosine levels can be induced by cyanide, dinitrophenol, opiate, AMPA, NMDA, kainate, and 5-HT receptor activation, forskolin, inflammatory stimuli like LPS and IL-1β and by blocking adenosine kinase and adenosine deaminase (Dunwiddie and Masino, 2001).

1.10 Physiological Functions of Adenosine

Adenosine affects numerous physiological functions of the central nervous system, cardiovascular system, respiratory system, renal and gastrointestinal systems, and the immune system (Fredholm et al., 2001; Ohta and Sitkovsky, 2001; Revan et al., 1996a; Stevens et al., 2002). The known functions of adenosine are summarized in Table 1.

When comparing the physiological effects of and experimental triggers for the production of adenosine, adenosine seems to play an overall beneficial and protective role in the body. For example, adenosine levels are increased by seizure and adenosine has been shown to have anticonvulsant properties. Adenosine levels are also increased in response to inflammatory stimuli yet the actions of adenosine appear to be anti-inflammatory. Increases in adenosine are also triggered by neurotransmitter receptor activation and adenosine can modulate both neurotransmitter release and neurotransmitter receptor function.

CNS	Antinociception (A_1) *(Fredholm et al., 2001)* Promotion of sleep (A_1) Inhibition of neuronal activity in cholinergic nuclei Retrograde synaptic messenger Regulation of cerebral blood flow (A_{2A}) Neuroprotection (A_1) Preconditioning (A_1, A_3) Anticonvulsant (A_1) *(Dunwiddie and Masino, 2001)* Myelination and oligodendrocyte differentiation *(Stevens et al., 2002)* Decreased neurotransmitter release (glutamate) Decreased locomoter activity (A_{2A}) *(Collis and Hourani, 1993)*
Immune System	Platelet aggregation (A_{2A}) Mast cell degranulation (A_{2B}, A_3) *(Fredholm et al., 2001)* Decreased neutrophil chemotaxis and killing (A_1, A_{2A}) *(Collis and Hourani, 1993; Revan et al., 1996)* Shift of TH1/TH2 dominance Decreased T lymphocyte adhesion
Cardiovascular	Regulation of blood pressure (A_{2A}) Regulation of vascular permeability (A_3) Preconditioning (A_1, A_3) Cardioprotection (A_{2A}, A_3) *(Fredholm et al., 2001)*
GI	Decreased acid secretion (A_1) *(Collis and Hourani, 1993)*
Metabolic	Decreased lipolysis (A_1) Increased insulin sensitivity (A_1) *(Collis and Hourani, 1993)*
Renal	Decreased glomerular filtration rate (A_1) Inhibition of renin release (A_1) *(Collis and Hourani, 1993)*
Respiratory	Bronchodilation (A_2) Bronchoconstriction (A_1) *(Collis and Hourani, 1993)*
Other	Regulation of pain (A_1) Lymphoma cell proliferation (A_3)

Table 1: Biological Functions of Adenosine

1.11 Adenosine Receptors

The actions of adenosine are mediated through interactions with 4 subtypes of cell surface adenosine receptors all of which have been identified and cloned. Adenosine A_1, A_{2A}, A_{2B}, and A_3 receptors are seven transmembrane domain receptors that are G-protein coupled. The physiological effects of adenosine receptor activation depend on the cell signaling events activated or mediated by those receptors. Each subtype of adenosine receptor is coupled to different G protein subunits and each receptor subtype activates different signaling pathways (Table 2).

Studying mice genetically deficient in adenosine receptors has helped understand their biological significance. Such studies were obviously aided by the finding that adenosine receptor deficient mice were viable, fertile, and developed normally. Two separate adenosine A_{2A} receptor knock-out mice have been generated; one targeted the first coding exon and the second targeted the second coding exon. Studies from these mice have implicated a role for adenosine A_{2A} receptors in mediating pain, inhibiting platelet aggregation, regulating blood pressure, regulating stimulant effects of caffeine on motor control, and mediating ischemic brain damage (Fredholm et al., 2001; Klinger et al., 2002). Mice genetically deficient in A_1 and A_3 receptors have also been generated, and have helped elucidate some of the biological functions of these receptors. A_3 receptor deficient mice exhibit decreased mast cell degranulation, decreased vascular permeability, and increased cardiovascular effects of

exogenously administered adenosine (Fredholm et al., 2001; Salvatore et al., 2000). Finally, studies of A_1 receptor deficient mice have implicated A_1 receptors in mediating anxiety, hyperalgesia, excitatory neurotransmission, hypoxia, and renin levels (Fredholm et al., 2001).

Receptor	G protein	Effector
A_1	$G_{i1/2/3}$, G_o	↓ **cAMP** ↑ PLC (IP_3/DAG) ↑ PLA_2 ↑ PLD ↑ K+ channels ↓ Ca^{2+} channels
A_{2A}	G_s, G_{olf}, $G_{15/16}$	↑ **cAMP** ↓ Ca^{2+} channels ↑ IP_3 ($G_{15/16}$)
A_{2B}	G_s	↑ **cAMP** ↑ PLC (IP_3/DAG)
A_3	G_{i3}, $G_{q/11}$	↓ **cAMP** ↑ PLC (IP_3/DAG) ↑ $[Ca^{2+}]_i$

Table 2: Summary of Adenosine Receptor Signaling. Abbreviations: phospholipase C (PLC), phospholipase A_2 (PLA_2), inositol trisphosphate (IP_3), diacylglycerol (DAG), phospholipase D (PLD) (Fredholm et al., 2001)

G proteins are composed of two subunits, the α subunit, which normally defines the type of G protein, and the $\beta\gamma$ subunit. Upon activation G protein subunits dissociate from the receptor complex and couple to effector proteins like adenylyl

cylase. Adenosine A_1 receptors are coupled to Gi proteins (subunit 1, 2 or 3) and Go proteins. Typically these receptors inhibit adenylyl cyclase, activate G-protein-dependent inwardly rectifying K^+ channels, inhibit Ca^{2+} channels and activate PLC (Fredholm et al., 2001; Klinger et al., 2002). In general, these signaling effects result in decreased synaptic transmission and hyperpolarization in the brain as well as sedation and anticonvulsant effects (Klinger et al., 2002). In the cardiovascular system, A_1 receptor activation has been shown to cause vasoconstriction and bradycardia. A_1 receptor activation decreases glomerular filtration rate and inhibits renin release in the kidney, causes bronchoconstriction in the lungs, and inhibits GI acid secretion (Collis and Hourani, 1993). A_1 receptors appear to participate in the short term protective effects of pre-conditioning in the brain and heart following hypoxia or ischemia (Klinger et al., 2002).

Adenosine A_{2A} receptor stimulation can result in activation of adenylyl cyclase, and may either activate and inactivate Ca^{2+} channels (Fredholm et al., 2001). A_{2A} receptors have been shown to both facilitate and inhibit neurotransmitter release. The physiological functions of A_{2A} receptors are less widespread than those of A_1 receptors and primarily involve effects on the immune system although A_{2A} receptors can be protective following ischemic and hypoxic insults by increasing local blood flow and stimulating endothelial cell growth and angiogenesis (Klinger et al., 2002). A_{2A} receptors are different from the other adenosine receptors in that they have an unusually long C-terminal tail

composed of 122 amino acids as compared to 36 amino acids for A_1 receptors in humans (Klinger et al., 2002). Although the function of the C-terminal tail is not well described, it is responsible for the tonic or constitutive activity of A_{2A} receptors, is rich in serine and threonine residues that are phosphorylated following PKC activation, is rich in proline residues which indicates possible interactions with SH3 domain proteins, and may contribute to coupling of A_{2A} receptors to downstream signaling effectors (Klinger et al., 2002).

Adenosine A_{2B} receptors have not been studied as extensively and consequently signaling events mediated by A_{2B} receptors are not as well characterized as the other subtypes of adenosine receptors. Like A_{2A} receptors, they activate adenylyl cyclase and also phospholipase C (PLC). The physiological functions of A_{2B} receptors are not well known but they participate in immune functions like mast cell degranulation (Klinger et al., 2002).

Adenosine A_3 receptors inhibit adenylyl cyclase, activate PLC and increase intracellular Ca^{2+} levels. In the brain, A_3 receptors have been shown to uncouple A_1 receptors, can potentially uncouple metabotropic glutamate receptors, and may trigger apoptosis in neurons (Abbracchio and Cattabeni, 1999; Fredholm et al., 2001; Klinger et al., 2002). Activation of A_3 receptors can mediate the immune system by stimulating mast cell degranulation and regulating cytokine production (Jacobson, 1998; Klinger et al., 2002; Sajjadi et al., 1996). A

summary of adenosine receptor signaling and physiological functions can be found in Tables 1 and 2.

1.12 Adenosine Receptor Affinity

The affinity of adenosine receptors for adenosine is quite variable and gives some insight into the receptor's physiological functions. A_1 receptor affinity is ~ 70 nM, A_{2A} receptor affinity is ~ 150 nM, A_{2B} receptor affinity is ~ 5100 nM, and A_3 receptor affinity is ~ 6500 nM (Dunwiddie and Masino, 2001). Interestingly, estimates of basal adenosine concentrations are in the range of 25-250 nM, concentrations that suggest a tonic activation of both A_1 and A_{2A} receptors (Dunwiddie and Masino, 2001). This suggests a strong role for basal adenosine levels in modulating neuronal excitability, cardiovascular function and immune responses given known physiological roles for these two receptors in these systems. Activation of A_{2B} and A_3 receptors occurs at elevated concentrations of adenosine, levels that are attained by stressful stimuli, suggesting the importance of these receptors during cell or tissue stress and diseases.

1.13 Adenosine and the Immune System

Several reports over the last decade have indicated anti-inflammatory and immune modulatory actions of adenosine. Adenosine is produced in increased levels during pathological conditions like stroke and tissue damage and these conditions are also associated with local immune and/or inflammatory responses. As a result, local production of adenosine may influence development of an

immune or inflammatory response. Initial studies addressing the adenosine – immune system connection identified the development of severe combined immunodeficiency syndrome in patients with a genetic deficiency in adenosine deaminase (Adanin et al., 2002; Blackburn, 2003). Adenosine deaminase deficiency resulted in decreased adenosine metabolism, increased adenosine levels, and an immune deficient phenotype. Thus, adenosine and adenosine receptors continue to be implicated as being important modulators of the immune system.

Subsequent studies on specific immune cells have elucidated at least some of the mechanisms involved in adenosine modulation of the immune system. To date, adenosine has been shown to influence the function of monocytes, macrophages, neutrophils and mast cells involved in innate immune responses and inflammation as well as T cells and dendritic cells involved in adaptive immune responses.

Adenosine, acting through A_2 receptors, decreases the production of free radicals and the ensuing respiratory burst of neutrophils (Revan et al., 1996; Sullivan et al., 1999). These effects can impair the killing mechanisms of neutrophils, ultimately decreasing the ability of these cells to destroy pathogens or cells infected with pathogens. Adenosine also influences the function of macrophages and monocytes primarily by modulating cytokine production. Adenosine increases production of IL-10, a cytokine involved in mediating TH2

and B lymphocyte (adaptive) responses (Hasko et al., 2002; Hasko et al., 1996; Le Moine et al., 1996). In addition, adenosine decreases production of TNF-α and IL-12, both cytokines involved in activation of phagocytes and TH1 immune responses (Hasko et al., 2002; Hasko et al., 2000; Hasko et al., 1996; Mayne et al., 2001). The cumulative effect of adenosine on neutrophils, monocytes, and macrophages may be decreased activation of and pathogen destruction by phagocytes, as well as decreased TH1, and increased TH2 and B lymphocyte-mediated immune responses.

Adenosine, acting primarily through A_3 receptors, can influence the function of mast cells (Jacobson, 1998; Klinger et al., 2002; Salvatore et al., 2000). Mast cells participate in innate immune responses and inflammation by releasing factors upon activation that increase vascular permeability and smooth muscle contraction (histamine), degrade basement membrane and activate complement (proteases), recruit neutrophils and eosinophils (chemotactic factors), and increase platelet activation and vascular permeability (clotting/kinin systems) (Nathan, 2002). Adenosine binds to A_3 receptors on mast cells and increases degranulation; degranulation normally mediated by IgE results in allergy and by complement C3a, C4a, and C5a results in acute inflammation. As a result, adenosine may contribute to symptoms of asthma or IgE-mediated allergy because the development of both disorders involves mast cell degranulation.

In addition to influencing inflammation and innate immunity, adenosine may also modulate adaptive immune responses through actions on dendritic cells and T cells. Adenosine increases macropinocytosis, chemotaxis, MHC1 expression, CD80, CD86, and CD54 expression on dendritic cells as well as decreasing TH1 polarization and IL-12 production (Panther et al., 2002). The cumulative effect on dendritic cells is increased maturation and activity, increased antigen presentation to cytotoxic T lymphocytes (via MHC1), increased T cell activation, and decreased TH1 polarization. Like the effects on phagocytes, adenosine appears to shift immune responses toward humoral immunity through its actions on dendritic cells.

Finally, adenosine decreases T cell proliferation and cytotoxic T lymphocyte activity. Specifically, adenosine acts on cytotoxic T lymphocytes to decrease proliferation/clonal expansion, decrease adhesion through $\alpha 4\beta 7$ integrin binding, decrease granule release, decrease protease, granzyme and perforin production, and decrease Fas-FasL cytotoxicity (Hoskin et al., 2002; MacKenzie et al., 2002; Mirabet et al., 1999). Adenosine also acts on CD4+ T helper cells to decrease proliferation, decrease TH1 responses by decreasing IL-12 and IFN-γ production, and increase TH2 responses by increasing IL-10 (Hoskin et al., 2002).

Although many specific effects have been studied on different cell types, these results together suggest that adenosine acts (1) to decrease cell mediated immunity by decreasing the activity of neutrophils, macrophages, and cytotoxic T

lymphocytes and (2) to promote humoral immunity by increasing antigen presentation, maturation, and activity of dendritic cells, as well as increasing TH2 mediated and decreasing TH1 mediated T cell responses. Consequently, adenosine may act to shape a particular type of immune response and because the specific effects of adenosine are mediated by different receptors, the overall effect may also be influenced by local levels of adenosine, given the differences in affinity of adenosine receptors for adenosine.

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Chapter 1

Adenosine Receptor Activation Inhibits TNF-α Protein Production

Abstract

Controlling pro-inflammatory cytokine production continues to be an active area of research towards combating immune disorders like rheumatoid arthritis, Crohn's disease and multiple sclerosis. The primary drugs used for multiple sclerosis are believed to work by altering the balance of cytokine production and promoting a TH2 dominant immune response. Drugs designed to specifically inhibit one cytokine, such as TNF-α neutralizing drugs, continue to be tested clinically for rheumatoid arthritis and Crohn's disease. The clinical focus on cytokine regulation emphasizes the importance of basic research to understand endogenous factors capable of controlling cytokines in particular and immune responses in general. Adenosine is a purine nucleoside produced in response to metabolism and stress and is one such factor that controls cytokine production. Adenosine levels are increased in response to inflammation and as such may modulate the developing immune response. Using phorbol esters phorbol-12-myristate-13-acetate (PMA) and phytohemagglutinin (PHA) to stimulate human pro-monocytic U937 cells, TNF-α protein levels were increased in cell culture supernatant in a linear fashion when treated at time intervals increasing from 30 minutes to 24 hours and production of TNF-α protein required de novo protein synthesis. Following cell stimulation for 4 hours, adenosine receptor agonists selective for A_1, A_{2A}, and A_3 receptors dose-dependently inhibited TNF-α protein production and the A_{2A} receptor agonist was the most potent and efficacious. Receptor-selective antagonists significantly reversed the anti-TNF-α effects of all three receptor agonists. Finally, the anti-TNF-α effects of the A_{2A} receptor

agonist were maintained following stimulation for 8 hours. These data suggest that adenosine receptors may contribute to modulation of developing immune responses by controlling production and release of the pro-inflammatory cytokine TNF-α in cells of monocytic lineage.

Introduction

Adenosine and adenosine receptors appear to play an important role in the regulation of immune and inflammatory responses. To date, studies have focused primarily on the role of adenosine and adenosine receptors in controlling cytokine production as a means of regulating immune and inflammatory responses. Much of the early studies focused on pro-inflammatory cytokines like TNF-α and investigated the role of both exogenous and endogenous adenosine in controlling the production of this cytokine. Through the use of specific adenosine receptor agonists, the contribution of specific adenosine receptors in controlling the production of cytokines like TNF-α was studied in macrophages, monocytes and cardiac cells. Since these initial studies were conducted, modulators of endogenous adenosine levels and specific adenosine receptor agonists have been shown to control TNF-α production both in vitro and in vivo. Adenosine and adenosine receptor agonists have been shown to control TNF-α production in several cells and tissues including rat cardiomyocytes (Wagner et al., 1999), failing human heart (Wagner et al., 1999), neonatal rat myocytes (Wagner et al., 1998), rat adrenal zona glomerulosa and zona fasciculata/reticularis cells (Ritchie et al., 1997), primary rat ovary cells (Ritchie et al., 1997), C6 glioma astrocyte cells (Brodie et al., 1998), U937 pro-monocytic cells (Mayne et al., 2001), THP-1 monocytic cells (Bshesh et al., 2002), primary human monocytes (Bouma et al., 1994; Le Moine et al., 1996), rat peritoneal macrophages (Ritchie et al., 1997), mouse peritoneal macrophages (Hasko et al., 2000), differentiated U937 macrophage cells (Sajjadi et al., 1996), RAW

264.7 macrophages (Firestein et al., 1994; Hasko et al., 1996), and in plasma from mice injected with LPS (Firestein et al., 1994; Hasko et al., 1996). These studies have identified anti-TNF-α effects of adenosine using adenosine itself or adenosine receptor agonists and both are added as drug treatments.

Endogenous adenosine is also capable of regulating TNF-α production. Using THP-1 cells, Bshesh and collegues showed that LPS-induced TNF-α production was augmented in the presence of the adenosine receptor antagonist 8-(3-chlorostyryl) caffeine (Bshesh et al., 2002) implicating a role of endogenous adenosine in regulating LPS-induced TNF-α production. LPS-stimulated THP-1 cells showed adenosine levels 6-fold higher than those in untreated cells after 4 hours, suggesting that endogenous adenosine levels increased by inflammatory stimuli may be able to control TNF-α production (Bshesh et al., 2002). Plasma levels of TNF-α were increased following LPS injection in mice and these increases in TNF-α were decreased when mice were treated with LPS in combination with an adenosine kinase inhibitor to decrease adenosine metabolism and increase endogenous adenosine levels (Firestein et al., 1994). Others found no significant effect of the adenosine deaminase inhibitor deoxycoformycin on TNF-α levels induced by LPS in primary human monocytes, suggesting that adenosine deaminase may not be the predominant pathway for adenosine mebabolism in those cells under those conditions (Bouma et al., 1994). Different affinities of adenosine kinase and adenosine deaminase for adenosine may explain the differences in anti-TNF-α effects of adenosine kinase

and adenosine deaminase inhibitors because the Km values for AK are 1-2 fold lower than those for ADA, suggesting that under physiological conditions AK is likely the dominant pathway of adenosine metabolism (Latini and Pedata, 2001).

The cell signaling mechanisms that mediate the anti-TNF-α effects of adenosine and adenosine receptors are poorly understood. Identifying the signaling mechanisms activated by these receptors that specifically control biological functions of adenosine like cytokine production may help identify new therapeutic targets for disorders that have an immune component. As a result, we investigated the anti-TNF-α effects of adenosine receptor agonists and determined signaling mechanisms by which adenosine A_{2A} receptor activation controls TNF-α production. The studies in this Chapter outline the development of our model and investigate the anti-TNF-α effects of A_1, A_{2A}, and A_3 receptors.

Methods

TNF-α Assays: U937 cells were cultured to a density of 800,000 cells/ml in RPMI growth media and were plated in 96 well plates with fresh media at a density of 800,000 cells/ml 1 hour prior to each experiment. As a control stimulus for the production and release of TNF-α, cells were treated with 10 ng/ml phorbol-12-myristate-13-acetate (PMA) and 5 μg/ml phytohemagglutinin (PHA) for 4 hours at 37°C. PMA is a phorbol ester that activates protein kinase C, a well-characterized signaling pathway that transmits signals in activated immune cells. PHA is a mitogen that stimulates cell proliferation and agglutination, both consequences of monocyte and lymphocyte activation.

To determine the effects of pre-treatment of U937 cells with adenosine receptor agonists on TNF-α protein production, adenosine receptor agonists and/or antagonists were prepared in RPMI media and added to cell cultures, unless otherwise indicated, 30 minutes prior to stimulation with PMA/PHA. Following the addition of PMA/PHA, cells were incubated for 4 hours, supernatants were collected, and TNF-α protein levels were determined by ELISA as described in the Appendix. For all chapters of this thesis, statistics were performed for each series of experiments as outlined in the Appendix. Data are presented as mean ± standard error and each figure is generated from a minimum of three individual experiments.

To determine the duration of the anti-TNF-α actions of adenosine A_{2A} receptors, the adenosine receptor agonist CGS 21680 and the adenosine receptor antagonist ZM 241385 were prepared in RPMI 1640 media and added to cells for 30 minutes. Cell cultures were then stimulated with PMA/PHA for increasing time intervals up to 24 hours and TNF-α protein levels were determined by ELISA.

Results

PMA/PHA induces increases in TNF-α levels in U937 cells. Although many cells in the body produce TNF-α, its main source is monocytes and macrophages. Our studies were designed to determine how adenosine receptor systems regulate TNF-α production. As a model system to study TNF-α, we used mitogens PMA and PHA to stimulate U937 cells to produce TNF-α protein. To begin our studies, we treated U937 cells with PMA/PHA for time intervals ranging from 1 to 24 hours and measured TNF-α protein production at each interval. Treatment of U937 cells with PMA/PHA for 1 hour produced no detectable increases in TNF-α. Significant increases were observed at 2 hours ($p < 0.05$) and TNF-α protein levels increased linearly up to 24 hours with maximum TNF-α production of 2.1 ng in 200 μl of cell culture supernatant (Figure 1.1). For all subsequent experiments, we stimulated U937 cells for 4 hours with PMA/PHA, a condition that produced 467 ± 104 pg of TNF-α in 200 μl of cell culture supernatant.

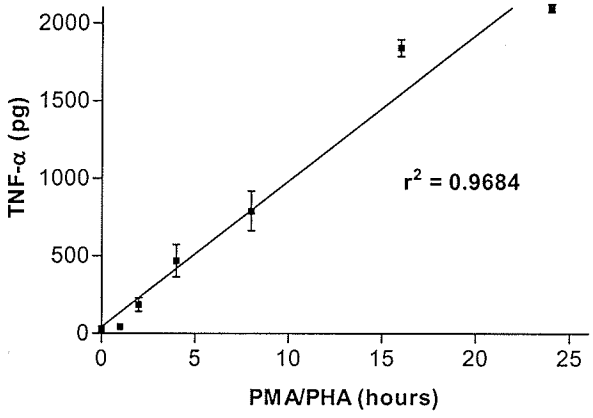

Figure 1.1: TNF-α production induced by PMA/PHA stimulation of U937 cells. U937 cells were treated with PMA/PHA for 1 to 24 hours. Supernatants were collected and TNF-α levels were determined by ELISA.

PMA/PHA-induced TNF-α production requires transcription and protein synthesis. Several groups have reported that cytokine production induced by inflammatory stimuli requires transcription and protein synthesis. Although TNF-α is produced as a precursor protein that resides in the plasma membrane prior to processing by TNF-α converting enzyme, stimulation of U937 cells with PMA/PHA for 30 minutes (data not shown) or 1 hour (Figure 1.1) did not produce any detectable increases in levels of TNF-α in cell culture supernatant. This suggests that U937 cells do not express precursor TNF-α in the plasma membrane that is available to be processed and released upon inflammatory stimulus. To test the hypothesis that PMA/PHA-induced TNF-α production requires transcription and protein synthesis, we pre-treated U937 cells with the transcription inhibitor actinomycin D or the protein synthesis inhibitor cycloheximide. Actinomycin D and cycloheximide both dose-dependently inhibited PMA/PHA-induced TNF-α production (Figure 1.2) and this suggests that PMA/PHA-induced TNF-α protein production requires transcription and protein synthesis.

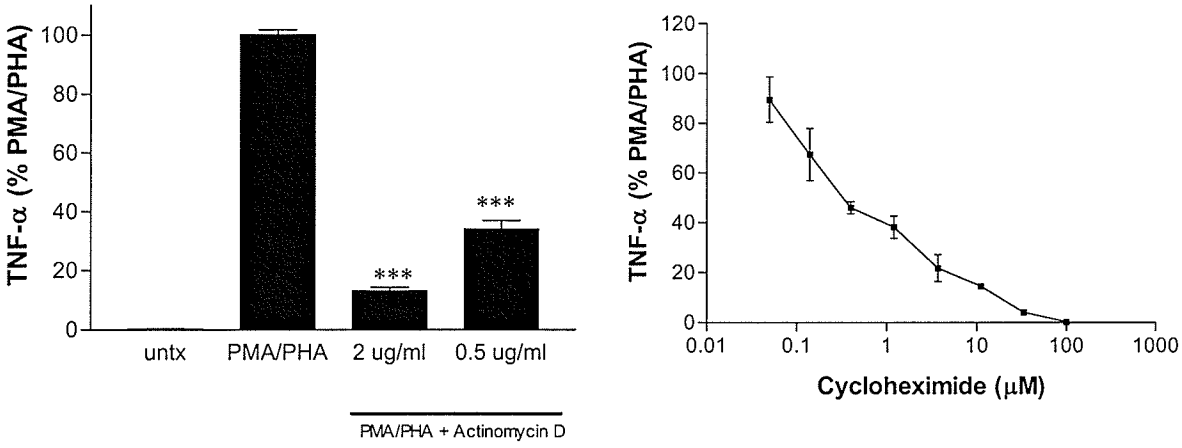

Figure 1.2: PMA/PHA-induced TNF-α production requires transcription and protein synthesis. U937 cells were pre-treated with the transcription inhibitor actinomycin D or the protein synthesis inhibitor cycloheximide for 15 minutes prior to PMA/PHA stimulation. After 4 hours, supernatants were collected at the indicated time points and TNF-α levels were determined by ELISA. *** $p < 0.001$ vs. PMA/PHA

Adenosine receptor agonists inhibit PMA/PHA-induced TNF-α production in U937 cells. Several groups have shown that adenosine and adenosine receptor agonists inhibit TNF-α production in macrophages and monocytes (Bouma et al., 1994; Bshesh et al., 2002; Hasko et al., 2002; Hasko et al., 2000; Hasko et al., 1996; Le Moine et al., 1996; Link et al., 2000; Mayne et al., 2001; Ohta and Sitkovsky, 2001; Sitkovsky, 2003). In order to verify that adenosine receptor agonists inhibited TNF-α production induced by PMA/PHA in U937 cells, and to determine the relative potencies and efficacies with which adenosine receptor agonists affected TNF-α levels, cell cultures were treated with agonists specific for adenosine receptors expressed by U937 cells (Mayne et al., 2001) prior to PMA/PHA stimulation (Figure 1.3). Pre-treatment with the specific adenosine

receptor agonists R-PIA (A_1), CGS 21680 (A_{2A}), and IB-MECA (A_3) for 30 minutes all significantly reduced TNF-α levels in a dose-dependent manner (Figure 1.3A). Maximum reduction of TNF-α levels was 94 ± 1% for CGS 21680, 86 ± 2% for R-PIA, and 76 ± 6% for IB-MECA. The apparent IC_{50} values for CGS 21680 were 46 ± 2 nM, for R-PIA were 161 ± 4 nM, and 614 ± 3 nM for IB-MECA. The effects of all three agonists (100 nM) on TNF-α levels were significantly reversed ($p < 0.01$) by their respective antagonists; 8-PT (A_1), ZM 241385 (A_{2A}) and MRS 1220 (A_3) (Figure 1.3B). Addition of fresh RPMI, the media used to dissolve the agonists and antagonists, produced non-significant 2% decreases in levels of TNF-α (data not shown).

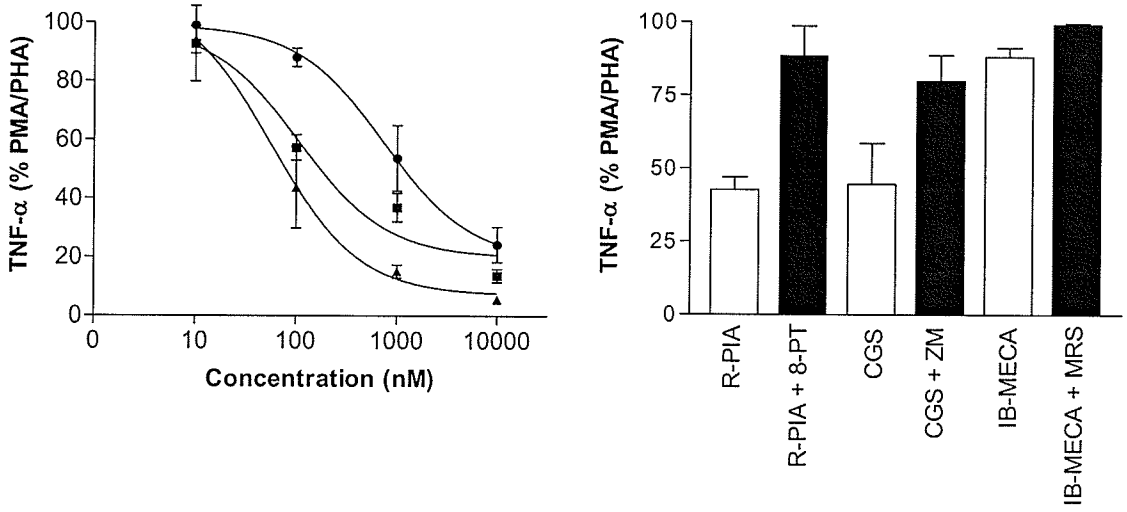

Figure 1.3: Effects of adenosine receptor activation on TNF-α levels in PMA/PHA-stimulated U937 cells. **A** Cells were pre-treated with the adenosine A_1 receptor agonist, R-PIA (squares), adenosine A_{2A} receptor agonist CGS 21680 (triangles), or the adenosine A_3 receptor agonist IB-MECA (circles) for 30 minutes prior to PMA/PHA stimulation for 4 hours. TNF-α levels in supernatants were determined by ELISA. **B** Receptor specific antagonists 8-PT (A_1), ZM 241385 (A_{2A}), and MRS 1220 (A_3) significantly reversed ($p < 0.05$) the reduction of TNF-α levels by respective agonists (100 nM of each agonist and antagonist).

Several groups have reported desensitization of adenosine receptors upon prolonged stimulation with receptor specific agonists and our group reported previously that the anti-TNF-α effect of A_{2A} receptors was lost when the receptor was activated with CGS 21680 for 18 hours prior to inflammatory stimulation (Mayne et al., 2001). In order to determine whether the anti-TNF-α effect of the adenosine A_{2A} receptor agonist would persist past 4 hours or whether it would be lost during long periods of immune cell activation, U937 cells were pre-treated with CGS 21680 for 30 minutes and stimulated with PMA/PHA for time intervals ranging from 2 to 24 hours. The anti-TNF-α effect of CGS 21680 was maintained for PMA/PHA stimulation at 2, 4, and 8 hours. Between 8 and 16 hours of PMA/PHA stimulation, the anti-TNF-α effect of CGS 21680 began to decrease and had disappeared completely by 24 hours (Figure 1.4).

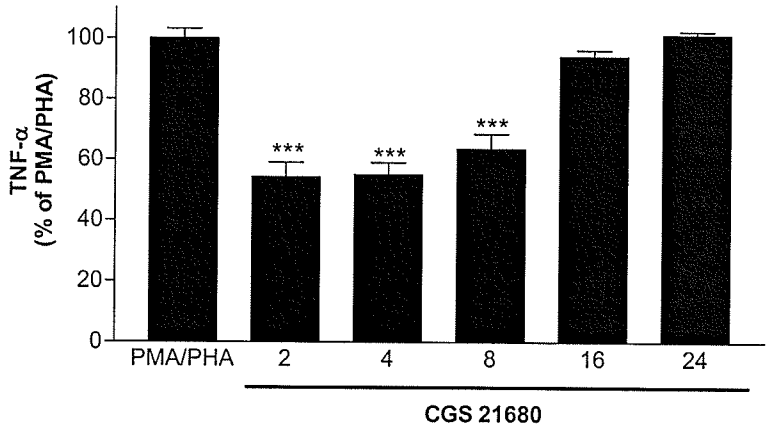

Figure 1.4: Effect of duration of immune cell activation on the anti-TNF-α effect of adenosine A_{2A} receptors. U937 cells were pre-treated with CGS 21680 (1 μM) for 30 minutes prior to PMA/PHA stimulation for increasing time intervals ranging from 2 to 24 hours. Supernatants were collected at the indicated time points and TNF-α levels were determined by ELISA. *** $p < 0.001$ vs. PMA/PHA

Discussion

Studies conducted by others and us have identified adenosine and adenosine receptor agonists as efficient regulators of TNF-α production. TNF-α plays a key role in immune responses and in inflammation by regulating the function of macrophages, monocytes, neutrophils, endothelial cells, and lymphocytes (Hasko et al., 2002), and as a regulator of TNF-α production adenosine receptors may be an important mediator of immune responses.

Although several studies investigating adenosine receptor effects on TNF-α have used receptor specific agonists, recent evidence suggests that most of these studies were conducted using drug concentrations that are not receptor specific. In fact, few studies have isolated receptor-specific anti-TNF-α effects using pharmacological approaches. Ritchie and collegues showed that CGS 21680, at an A_{2A} receptor specific concentration, blocked TNF-α production in rat adrenal zona glomerulosa cells (Ritchie et al., 1997). Using U937 cells differentiated into macrophages with PMA, Sajjadi et al, showed that activation of A_3 receptors reduced LPS-induced TNF-α production (Sajjadi et al., 1996). In addition, studies with adenosine A_{2A} receptor deficient mice have confirmed that A_{2A} receptors exert anti-TNF-α effects on LPS-induced peritoneal macrophages (Hasko et al., 2000).

Even though others have shown anti-TNF-α effects of adenosine receptor agonists in monocytes and macrophages, many of the agonists tested were not

receptor selective. As a first step towards identifying cell signaling mechanisms mediating the anti-TNF-α effects of adenosine A_{2A} receptor activation, we established a model for studying TNF-α production in U937 cells and studied the anti-TNF-α effects of A_1, A_{2A} and A_3 receptor agonists. To establish our model, we stimulated undifferentiated U937 cells with PMA/PHA for time intervals ranging from 30 minutes to 24 hours and measured TNF-α levels by ELISA. We found that PMA/PHA-induced increases in TNF-α were linear up to 24 hours and chose the 4 hour time interval for all subsequent experiments. Although other groups have used longer time intervals, typically 24 hours in monocytes and macrophages (Bshesh et al., 2002; Hasko et al., 2000; Hasko et al., 1996; Le Moine et al., 1996), high levels of TNF-α can induce cell death and therefore we chose a time point within the linear portion of the curve showing TNF-α protein production without effects on U937 cell viability. In addition, prolonged treatment of U937 cells with PMA can induce differentiation. Our experiments were conducted at time intervals prior to significant differentiation of U937 cell cultures into macrophage phenotypes.

We showed that R-PIA, CGS 21680, and IB-MECA dose-dependently blocked PMA/PHA-induced TNF-α production. The apparent IC50 for R-PIA was 161 nM and was consistent with actions on A_1 and A_3 receptors (Klotz, 2000). The apparent IC50 value for CGS 21680 was 46 nM, which is consistent with actions on A_{2A} receptors (Klotz, 2000). The apparent IC_{50} for IB-MECA was 614 nM and is consistent with actions on both A_1 and A_3 receptors. The actions of CGS

21680 were reversed by ZM 214385 at a concentration selective for A_{2A} receptors, confirming the receptor specificity of CGS 21680 actions on TNF-α (Klotz, 2000). MRS 1220 reversed the actions of IB-MECA at a concentration selective for A_{2A} and A_3 receptors, confirming that IB-MECA actions on TNF-α were mediated by A_3 receptors since the IC50 for IB-MECA was selective for A_1 and A_3 (Klotz, 2000).

The observation that A_1, A_{2A}, and A_3 receptor activation each control TNF-α production suggests redundancy within the adenosine system for modulation of cytokine levels and raises questions as to the biological rationale for cells to express 3 adenosine receptors to control TNF-α. As discussed in the General Introduction, each adenosine receptor modulates the function of hematopoetic cells involved in different stages of a developing immune response suggesting distinct big-picture roles for each receptor subtype. It may be that TNF-α participates at multiple steps of a developing immune response, requiring different adenosine receptor subtypes to control its production in order to modulate individual stages of an immune response.

Finally, we showed that CGS 21680 blocked PMA/PHA-induced TNF-α production following stimulation of U937 cells up to 8 hours. CGS 21680 was unable to block TNF-α production if cells were stimulated for 16 or 24 hours with PMA/PHA. This finding is somewhat different from observations of others that CGS 21680 blocked TNF-α production in C6 glioma cells following stimulation

with LPS/IFN-γ for 48 hours (Brodie et al., 1998) and in THP-1 cells following stimulation with LPS for 24 hours (Bshesh et al., 2002). CCPA blocked TNF-α production in RAW 264.7 macrophages following stimulation with LPS for 24 hours (Hasko et al., 1996) and 2-chloroadenosine blocked TNF-α production in RAW 264.7 cells following stimulation with LPS for 24 hours (Firestein et al., 1994). Adenosine inhibited TNF-α production in human monocytes stimulated with either LPS or hydrogen peroxide for 24 hours (Le Moine et al., 1996). Finally, adenosine, CGS 21680, and IB-MECA blocked TNF-α production in peritoneal macrophages stimulated with LPS for 24 hours (Hasko et al., 2000).

Other studies in U937 cells showed TNF-α inhibition by adenosine receptor agonists for up to 24 hours. These studies were conducted in U937 cells that were stimulated with PMA and began to differentiate following 8 hours, indicated by increased expression of macrophage markers such as CD14 and CD68 and adoption of macrophage-like morphology (Sajjadi et al., 1996). In our model using undifferentiated U937 cells, CGS 21680 inhibited TNF-α production for up to 8 hours and the anti-TNF-α effects were lost following 16 and 24 hour PMA/PHA stimulation. The loss of anti-TNF-α effect may have resulted from receptor desensitization that has been shown in this model following 18 hour pretreatment of U937 cells with CGS 21680 (Mayne et al., 2001) or from changes in adenosine receptor expression during the initial stages of differentiation of U937 cells to macrophages. Other studies showing inhibition of TNF-α after 24 hours have used an A_1/A_3 receptor agonist or adenosine which activates all 4

adenosine receptors. Our studies have shown effects of CGS 21680 that are completely reversed by the A_{2A} receptor specific agonist ZM 241385, indicating primarily A_{2A} receptor actions.

Anti-TNF-α effects of adenosine and adenosine receptors may be especially important during conditions associated with excessive TNF-α production and during conditions associated with increased adenosine production. For example, multiple organ failure results from disorders like sepsis and is associated both with over-production of pro-inflammatory cytokines and increased systemic adenosine levels (Hasko et al., 2002). Multiple organ failure is initiated by a large scale systemic inflammatory response and is subsequently associated with macrophage and T cell dysfunction resulting from deficient production of pro-inflammatory cytokines like TNF-α by macrophages and shift in TH1/TH2 balance towards TH2 dominant immune responses. Dysfunction in macrophage and T cell responses result in deficient responses mounted towards the pathogen involved in the initial insult, causing serious infection and multiple organ failure (Hasko et al., 2002). Interestingly, the failure of macrophages to produce sufficient levels of pro-inflammatory cytokines and a shift towards TH2 dominant T cell responses occurs in conjunction with systemic adenosine levels increased from below 1 μM to levels estimated between 4-10 μM (Hasko et al., 2002). These results suggest that a sustained increase in adenosine levels associated with the initial inflammatory responses contributes to dysfunction of macrophages and a TH2 dominant immune response that prevents an adequate response to

the invading pathogen. Importantly, a recent study reported that adenosine has no immunosuppressive effects on macrophages until concentrations reach 3 μM (Hasko et al., 2000) and adenosine levels have been measured as high as 100 μM during an inflammatory response (Hasko et al., 2002). These data suggest that given adenosine receptor affinity for adenosine, A_1 and A_{2A} receptors may be involved in tonic control of cytokine production, whereas A_{2B} and A_3 receptors are involved only in inflammatory states associated with high adenosine levels that have immunosuppressive effects on macrophages.

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Chapter 2

Adenosine A_{2A} Receptor Activation Regulates TNF-α Post-Transcriptionally

Abstract

Production and release of pro-inflammatory cytokines from activated immune cells requires de novo protein synthesis and depends on activation of transcription factors like NF-κB to drive cytokine gene expression. Activation of monocytes and macrophages by inflammatory stimuli like lipopolysaccharide induces mitogen-activated protein kinase (MAPK) cell signaling pathways which activate NF-κB, ATF-2 and cJun, all transcription factors with binding sites on the TNF-α promoter. As a result, we hypothesized that regulation of TNF-α protein production by adenosine A_{2A} receptors would involve inhibition of transcription factor activity and induction of TNF-α gene expression. We showed that PMA/PHA-stimulation of U937 cells increased NF-κB binding activity and that NF-κB was required for TNF-α production but the A_{2A} receptor agonist did not decrease PMA/PHA-dependent induction of NF-κB. In addition, PMA/PHA did not increase phosphorylation of either ATF-2 or cJun, an indicator of their activity level. Activation of adenosine A_{2A} receptors decreased TNF-α levels post-transcriptionally because the A_{2A} receptor agonist did not inhibit PMA/PHA induction of TNF-α gene expression but inhibited TNF-α protein production when added up to 1 hour after PMA/PHA stimulation, a time point that coincides with presence of TNF-α mRNA but undetectable levels of TNF-α protein. Finally, A_{2A} receptor activation decreased TNF-α mRNA stability and half-life from 80 minutes to 37 minutes. These data suggest that adenosine A_{2A} receptors control TNF-α protein production post-transcriptionally at least in part by decreasing TNF-α mRNA stability and half-life.

Introduction

Production of TNF-α initiated by inflammatory stimuli requires transcription, translation, and processing. Pre-transcriptional regulation of TNF-α occurs by blocking gene expression through modulation of transcription factor activity. Post-transcriptional regulation of TNF-α involves modulation of mRNA stability and half-life, regulation of proteins involved in translation, and/or regulation of cell membrane processing by TNF-α converting enzyme. Transcription of the TNF-α gene is driven by activation of transcription factors that have binding sites on the TNF-α promoter. Transcription and translation of TNF-α is initiated by many stimuli like PMA and LPS which activate cell signaling cascades that ultimately converge on activation of transcription factors which can bind to the TNF-α promoter and drive TNF-α expression including NF-κB, ATF-2, Elk-1, Ets, Egr-1, NFAT, SP1, cJun and CREB (Falvo et al., 2000; Tsai et al., 2000; Tsai et al., 1996).

One of the most well studied transcription factors involved in TNF-α production, as well as transcription of other proteins involved in regulating immune responses, is NF-κB. NF-κB is a ubiquitously expressed transcription factor, which resides in cell cytoplasm as a dimer composed of proteins belonging to the Rel family. Most commonly found as a p65-p50 heterodimer, NF-κB is bound to its inhibitor protein IκBα in the cytoplasm under basal conditions, keeping the complex in an inactive state. Upon activation by stimuli like LPS and PMA, IκBα is phosphorylated via activation of NF-κB inducing kinase (NIK) and IκBα kinase

(IKK). Once phosphorylated, IκBα is released from NF-κB, ubiquitinated and degraded. Release of the inhibitor protein allows the NF-κB dimer to translocate to the nucleus and induce the expression of cytokines, chemokines, growth factors and anti-apoptotic proteins (Figure 2.1) (Ghosh and Karin, 2002). Recently adenosine has been shown to decrease NF-κB binding activity in the heart (Li et al., 2000). As a result, we hypothesized that by blocking NF-κB binding activity, adenosine may block TNF-α gene expression and thereby effectively regulate TNF-α protein production.

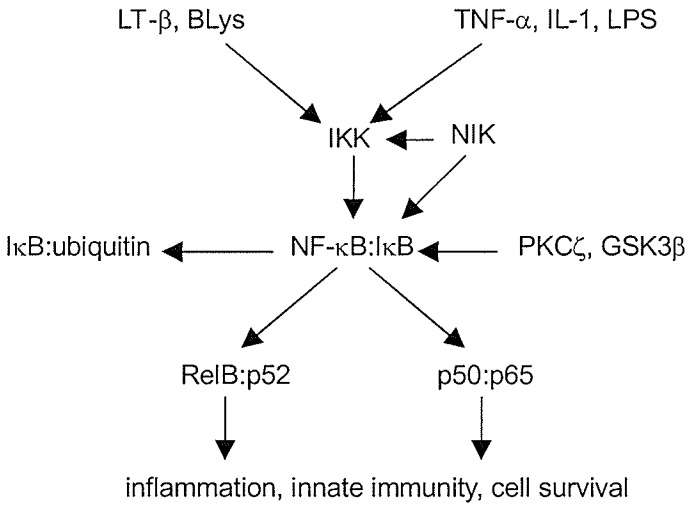

Figure 2.1: Activation of NF-κB. Cell signaling pathways activating NF-κB transcriptional activity. IKK - IκB kinase, NIK – NF-κB inducing kinase, PKC – protein kinase C, GSK3 – glycogen synthase kinase 3

ATF-2 is another transcription factor that also regulates TNF-α production. Normally, ATF-2 is found in the nucleus and has a low level of transcriptional activity because the DNA-binding domain at the C terminal of the protein is physically associated with the N-terminal transactivating domain. Activation of this transcription factor relies on phosphorylation of the N terminus, which

relieves the intramolecular inhibition and promotes transcriptional activity (Fuchs et al., 2000). Inflammatory signals activate MAPK signaling proteins like JNK and p38, both of which translocate to the nucleus upon activation and phosphorylate ATF-2 on serine residues 69 and 71 at the N terminus (Fuchs et al., 2000). Transcriptional activity of ATF-2 is dependent on phosphorylation by JNK or p38 (Tsai et al., 2000). Activation of ATF-2 also signals its ubiquitin-dependent degradation as negative feedback control. ATF-2 phosphorylation blocks its degradation in vitro but the association of active ATF-2 with cJun to form a transcriptionally active heterodimer facilitates ubiquitination and degradation (Fuchs et al., 2000). Ubiquitination of ATF-2 also occurs when the protein is dephosphorylated, which may be mediated by serine/threonine protein phosphatases PP1 and PP2A (Fuchs et al., 2000). Active ATF-2 transcriptional complexes induce expression of genes like TNF-α only when ATF-2 is phosphorylated. Stimulation of the murine macrophage cell line J774 with LPS induces the transcription of TNF-α in a manner dependent on ATF-2 (Tsai et al., 2000). When active in the nucleus, ATF-2 can complex with active cJun and bind to a cAMP response element (CRE) site on the TNF-α promoter. cJun is constitutively present in cells in an inactive form and like ATF-2, cJun is activated by phosphorylation, although primarily by JNK on serine residues 63 and 73 (Shanley et al., 2001). Activated cJun can also interact with cFos to form the AP-1 transcription factor. Both cJun heterodimers have been implicated in regulating TNF-α production.

Production of TNF-α in U937 cells induced by PMA/PHA requires transcription and protein synthesis (see Figure 1.2). As a result, we hypothesized that activation of adenosine A_{2A} receptors decreased TNF-α production pre-transcriptionally by blocking activation of transcription factors involved in production of TNF-α and subsequently inhibiting gene expression. Many studies have identified NF-κB as a transcription factor essential for induction of TNF-α gene expression by interacting with the TNF-α promoter. In addition, ATF-2:cJun heterodimers can bind to CRE binding sites on the TNF-α promoter and the activity of this transcriptional complex is regulated by p38 and JNK signaling pathways, which have been identified as important MAPK signaling molecules mediating TNF-α production in monocytes and macrophages (Fuchs et al., 2000; Rao, 2001; Tsai et al., 1996). Consequently, our experiments were designed to identify effects of adenosine A_{2A} receptor activation and PMA/PHA stimulation on NF-κB binding activity and phosphorylation of ATF-2 and cJun.

Methods

To determine if PMA/PHA-induced TNF-α production required NF-κB production, U937 cells were pre-treated for 30 minutes with SN50, a cell-permeable NF-κB inhibitor. Cell cultures were then stimulated with PMA/PHA for 4 hours, supernatants were collected and TNF-α production was measured by ELISA.

To determine if adenosine A_{2A} receptor activation decreased PMA/PHA-induced NF-κB binding activity, U937 cells were pre-treated for 30 minutes with CGS 21680 prior to stimulation with PMA/PHA for 2 hours. Cells were collected on ice, protein extracts isolated and NF-κB binding activity was determined by electrophoretic mobility shift assay as described in the Appendix.

To determine if adenosine A_{2A} receptor activation decreased PMA/PHA-induced phosphorylation and activation of cJun and ATF-2, U937 cells were stimulated with PMA/PHA for time intervals ranging from 5 to 60 minutes. Cells were collected on ice, protein extracts were prepared using a phosphorylation lysis buffer, and proteins were separated using SDS polyacrylamide gel electrophoresis as described in the Appendix. Phospho-cJun and phospho-ATF-2 levels were determined by western blot using phosphorylation state-specific antibodies against cJun and ATF-2 as described in the Appendix.

To determine if adenosine A_{2A} receptor activation decreased TNF-α production pre-transcriptionally, the adenosine receptor agonist CGS 21680, and the

adenosine receptor antagonist ZM 241385, were prepared in RPMI 1640 media and added to cells 30 minutes prior to stimulation with PMA/PHA for periods ranging from 30 minutes to 4 hours. Supernatants were collected and levels of TNF-α were determined by ELISA. Cell pellets were collected on ice from the same experiments and TNF-α mRNA levels were determined by RT-PCR as described in the Appendix.

To determine if the anti-TNF-α actions of adenosine A_{2A} receptors were affected by the timing of agonist addition, CGS 21680 and/or the adenosine receptor antagonist ZM 241385 were prepared in RPMI 1640 media and added to cells 30 minutes prior to, simultaneously with, or 30 minutes, 1, 2, or 3 hours after stimulation with PMA/PHA. Following the addition of PMA/PHA, cells were incubated for 4 hours, supernatants were collected, and levels of TNF-α were determined by ELISA.

To determine if CGS 21680 affected TNF-α mRNA stability, U937 cells were treated with PMA/PHA alone or in combination with CGS 21680 for 2 hours. Cells were collected at time 0 and remaining cultures were treated with actinomycin D to stop further transcription. Following actinomycin D treatment, cells were collected at time intervals increasing from 10 to 60 minutes. TNF-α mRNA levels were determined at each time point by semi-quantitative RT-PCR.

Results

CGS 21680 does not block PMA/PHA-induced increases in NF-κB binding activity. To determine if NF-κB activity is required for PMA/PHA-induced TNF-α production, U937 cells were treated with the cell permeable NF-κB inhibitor SN50 prior to PMA/PHA stimulation. SN50 is an NF-κB peptide inhibitor designed to bind to the nuclear localization sequence on active p65-p50 heterodimers in cell cytoplasm and thereby block translocation of this protein complex to the nucleus where it binds to the TNF-α promoter and initiates gene expression (Lin et al., 1995). SN50 completely inhibits NF-κB binding activity at 18 μM in endothelial cells (Lin et al., 1995) and inhibits 70% of TNF-α produced in murine macrophages at the same concentration (Ropert et al., 2001). SN50 inhibited PMA/PHA-induced increases in TNF-α levels with a statistically significant inhibition of 34 ± 5% ($p < 0.001$) observed with 20 μM (Figure 2.2A). These findings indicate that NF-κB was involved in PMA/PHA-induced production of TNF-α. In order to ensure that the anti-TNF-α effect of SN50 was due to blocking NF-κB, we stimulated U937 cells with PMA/PHA in the presence of the SN50 inactive peptide and measured TNF-α protein production in cell culture supernatants by ELISA. At concentrations of SN50 that significantly ($p < 0.001$) blocked TNF-α production, there was no significant anti-TNF-α effect by the SN50 inactive peptide (Figure 2.2B).

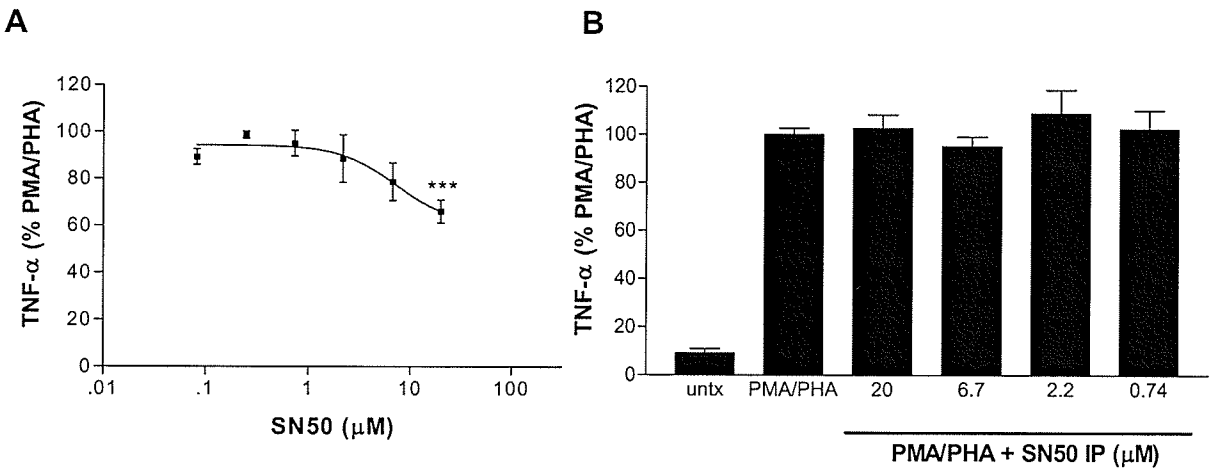

Figure 2.2: PMA/PHA-induced TNF-α production requires NF-κB. U937 cells were pre-treated with SN50 (A) or SN50 inactive peptide (SN50 IP) (B) for 30 minutes prior to stimulation with PMA/PHA for 4 hours. Supernatants were collected and TNF-α levels were determined by ELISA. *** $p < 0.001$ vs. PMA/PHA

To determine if activation of adenosine A_{2A} receptors would decrease PMA/PHA-induced increases in NF-κB binding activity as a mechanism for blocking TNF-α production, U937 cells were treated with CGS 21680 prior to stimulation with PMA/PHA for 30 minutes and NF-κB binding activity was determined by EMSA. PMA/PHA-induced increases in NF-κB binding activity were not inhibited by pre-treatment of U937 cells with CGS 21680 nor was the activity affected by ZM 241385 (Figure 2.3).

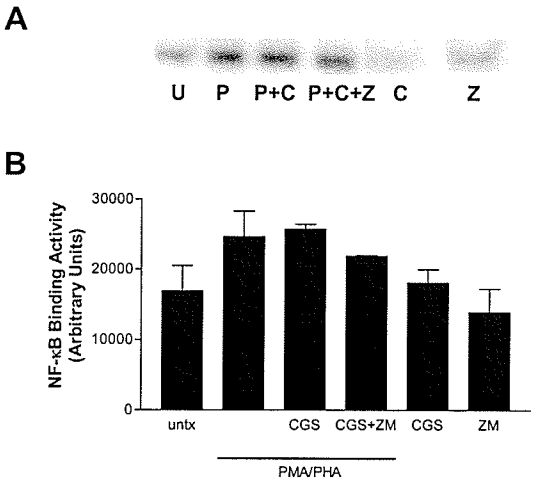

Figure 2.3: Adenosine A_{2A} receptor activation does not decrease PMA/PHA-induced NF-κB binding activity. U937 cells were pre-treated with CGS 21680 for 30 minutes prior to PMA/PHA stimulation. Cells were incubated with PMA/PHA for 30 minutes and NF-κB binding activity was determined by EMSA. **A** Representative gel showing untreated cells (U), cells treated with PMA/PHA (P) alone or in combination with CGS 21680 (C; 1 μM)) or CGS 21680 in combination with ZM 241385 (Z; 100 nM). **B** Data of 4 experiments shown graphically.

PMA/PHA does not induce transcription of TNF-α by activating ATF-2/cJun. We sought next to determine whether other transcription factors activated by PMA/PHA that initiate TNF-α gene expression may be targets of adenosine A_{2A} receptor activation. The TNF-α promoter contains binding sites for the transcription factors ATF-2 and cJun, and these proteins are involved in driving TNF-α gene expression in macrophages. ATF-2 and cJun are phosphorylated and activated by JNK and p38, both members of the MAPK signaling family known to be essential for TNF-α production (Rao, 2001; Zhu et al., 2000). When these proteins are phosphorylated, they form heterodimers and interact with the ATF-2 binding sites on the TNF-α promoter. As a result, we hypothesized that CGS 21680 blocks PMA/PHA-induced TNF-α production by decreasing the phosphorylation of ATF-2 and/or cJun. Accordingly, we stimulated U937 cells

with PMA/PHA for time intervals ranging from 5 to 60 minutes and measured phospho-ATF-2 and phospho-cJun levels by western blot. Stimulation of cell cultures with PMA/PHA did not increase phospho-ATF-2 protein levels over those detectable levels observed in untreated cells following stimulation for time intervals from 5 to 60 minutes (Figure 2.4). In addition, no detectable phospho-cJun levels were observed in untreated cell cultures or in cultures treated with PMA/PHA for any of the time points tested (data not shown). Because PMA/PHA did not increase phospho-cJun or phospho-ATF-2 levels over basal levels in untreated cells, we did not test whether phosphorylation of these proteins was blocked by CGS 21680 as a mechanism for controlling TNF-α production.

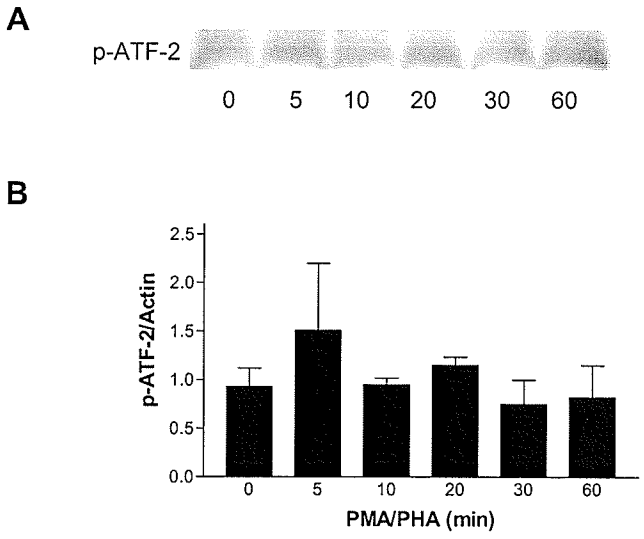

Figure 2.4: PMA/PHA does not affect levels of phospho-ATF-2. U937 cells were treated with PMA/PHA for time intervals increasing from 5 to 60 minutes and phospho-ATF-2 and actin protein levels were determined by western blot. **A** Representative western blot showing phospho-ATF-2 protein levels. **B** Results from three independent experiments are shown graphically.

CGS 21680 inhibited PMA/PHA-induced increases in TNF-α protein levels but not mRNA levels. Because transcription factors known to be involved in driving TNF-α expression in macrophages appeared not to be involved in A_{2A} receptor mediated inhibition of TNF-α production, we designed experiments to determine whether CGS 21680 inhibited PMA/PHA-induced increases in levels of TNF-α pre- or post-transcriptionally. Levels of TNF-α mRNA were measured in cell pellets from U937 cells and levels of TNF-α protein were measured in supernatants. For these experiments, cells were incubated with PMA/PHA for time periods of 30 minutes, 1, 2 and 4 hours. CGS 21680 inhibited production of TNF-α by 38 ± 7 % with 2 hr (data not shown) and by 42 ± 7 % ($p < 0.01$) with 4 hr incubations with PMA/PHA (Figure 2.5). TNF-α protein production was not detectable following 30 min or 1 hr incubations with PMA/PHA (data not shown). ZM 241385 (100 nM) completely reversed the inhibition of PMA/PHA-induced increases in TNF-α by CGS 21680 (Figure 2.5).

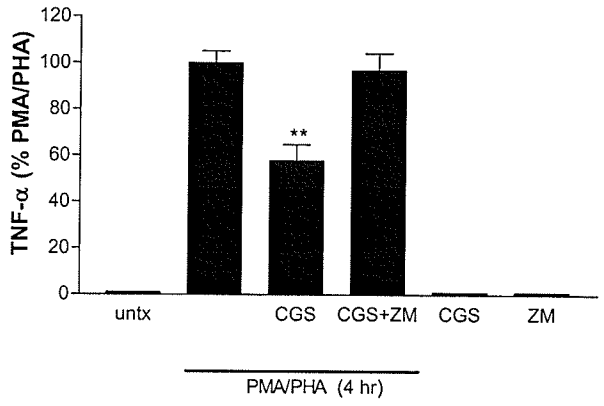

Figure 2.5: Adenosine A_{2A} receptor activation inhibits PMA/PHA-induced TNF-α production. U937 cells were pre-treated with CGS 21680 (1 μM) alone or in combination with ZM 241385 (100 nM) for 30 minutes prior to PMA/PHA stimulation. Cells were incubated with PMA/PHA for 4 hours, supernatants were collected and TNF-α levels were determined by ELISA. ** $p < 0.01$ vs. PMA/PHA

In cell pellets from these same samples, PMA/PHA-induced increases in TNF-α mRNA levels were found not to be inhibited by CGS 21680 at incubation times of 30 min, 1 hr, 2 hr (data not shown) and 4 hr (Figure 2.6). Treatment of U937 cells with CGS 21680 or ZM 241385 in the absence of PMA/PHA did not affect mRNA or protein levels of TNF-α (Figure 2.5 and 2.6).

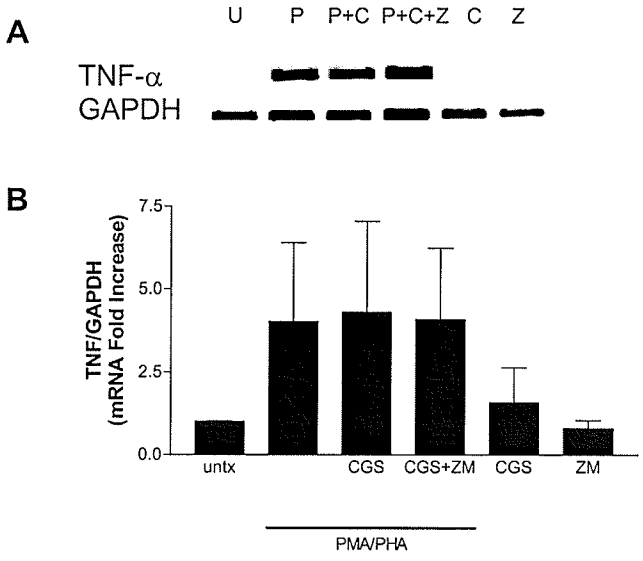

Figure 2.6: Adenosine A_{2A} receptor activation does not inhibit PMA/PHA-induced increases in TNF-α mRNA levels. U937 cells were pre-treated with CGS 21680 (1 μM) alone or in combination with ZM 241385 (100 nM) for 30 minutes prior to PMA/PHA stimulation. Cells were incubated with PMA/PHA for 4 hours, cells were collected and TNF-α mRNA levels were determined by RT-PCR. **A** Representative RT-PCR products imaged using ethidium bromide and agarose gel electrophoresis. **B** Results from 4 experiments expressed graphically.

To ensure that RT-PCR for TNF-α performed at 25 cycles produced PCR product within the linear range of our PCR reaction, we carried out RT-PCR with TNF-α primers at increasing cycle numbers with cDNA from PMA/PHA treated U937 cells. Twenty-five cycles produced RT-PCR product within the linear range of our PCR amplification curve (Figure 2.7).

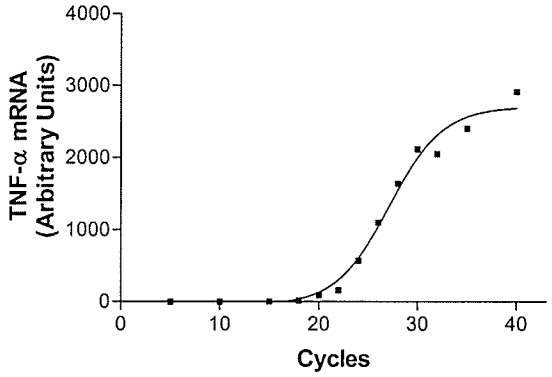

Figure 2.7: PCR amplification of TNF-α from U937 cells treated with PMA/PHA. U937 cells were treated with PMA/PHA for 4 hours. TNF-α mRNA levels were determined using RT-PCR for 5 to 40 cycles.

Adenosine A_{2A} receptor activation inhibited TNF-α production if added up to 1 hour after PMA/PHA stimulation. Pre-treatment of U937 cells with CGS 21680 appeared to block PMA/PHA-induced increases in TNF-α protein post-transcriptionally and TNF-α protein was not detectable in cell culture supernatants until 2 hours after PMA/PHA stimulation (Figure 1.1). Consequently, we hypothesized that CGS 21680 added simultaneously with or after PMA/PHA stimulation would result in an anti-TNF-α effect similar to that seen with a 30 minute agonist pre-treatment. To address this question, we compared the anti-TNF-α effects when CGS 21680 was added 30 minutes prior to, simultaneously with, or 30 minutes, 1, 2, or 3 hours after PMA/PHA stimulation. PMA/PHA-induced increases in TNF-α protein production were significantly inhibited ($p < 0.001$) by CGS 21680 if added simultaneously with, 30 minutes or 1 hour after PMA/PHA stimulation (Figure 2.8). The anti-TNF-α effect

of CGS 21680 was lost if added 2 or 3 hours after PMA/PHA stimulation, a time frame that coincided with detection of TNF-α protein in cell supernatants (Figure 1.1 and 2.8).

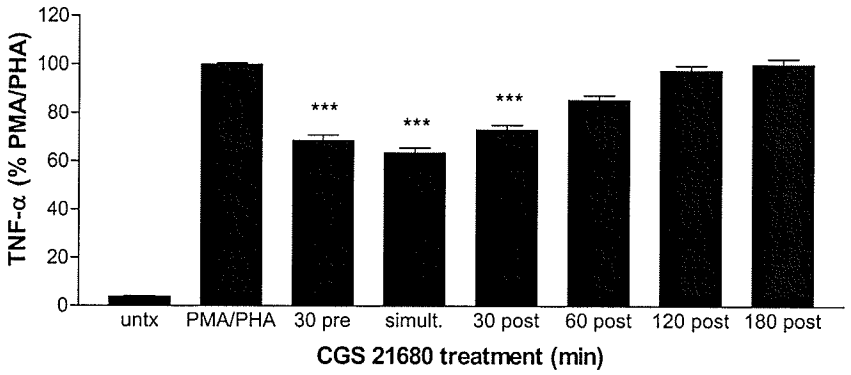

Figure 2.8: Activation of adenosine A_{2A} receptors inhibited PMA/PHA-induced TNF-α production when added up to 1 hour after PMA/PHA stimulation. U937 cells were treated with CGS 21680 (1 μM) 30 minutes prior to, simultaneously with, or 30 minutes, 1, 2, or 3 hours after PMA/PHA. Following stimulation with PMA/PHA for 4 hours, supernatants were collected and TNF-α levels were determined by ELISA. *** $p < 0.001$ vs. PMA/PHA

Adenosine A_{2A} receptor activation decreased TNF-α mRNA stability and half-life.

Although CGS 21680 did not inhibit PMA/PHA-induced increases in steady-state TNF-α mRNA levels, steady-state mRNA levels reflects a balance between mRNA degradation and gene transcription. As a result, changes in transcription or degradation and stability may not be reflected in steady-state mRNA levels. Because CGS 21680 did not inhibit initiation of TNF-α gene expression induced by PMA/PHA or activation of transcription factors involved in regulating TNF-α production, we tested the hypothesis that adenosine A_{2A} receptor activation

blocks TNF-α production by decreasing mRNA stability and half-life. We found that PMA/PHA-stimulated U937 cells showed progressively decreasing mRNA levels following addition of actinomycin D and initial significant ($p < 0.05$) mRNA decreases were observed at 60 minutes (Figure 2.9). In cells treated with CGS 21680 in combination with PMA/PHA, TNF-α mRNA levels decreased faster following addition of actinomycin D with initial significant ($p < 0.01$) mRNA decreases observed at 30 minutes, suggesting that activation of adenosine A_{2A} receptors decreases stability of PMA/PHA-induced TNF-α mRNA levels (Figure 2.9).

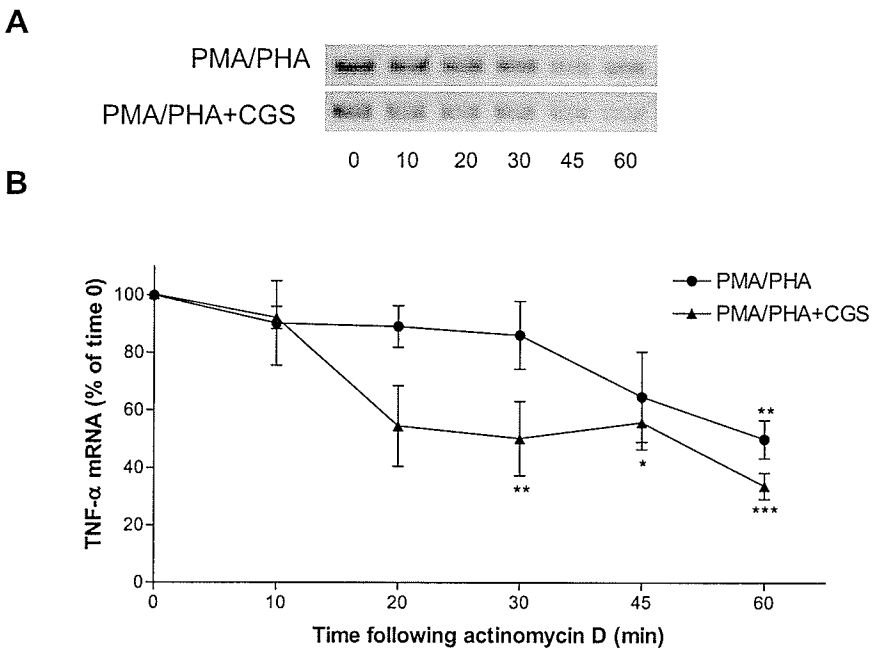

Figure 2.9: Adenosine A_{2A} receptor activation decreases TNF-α mRNA stability. A U937 cells were treated with PMA/PHA alone or in combination with CGS 21680 (1 μM) for 2 hours. Actinomycin D (5 μM) was added at time 0 and cells were collected at time intervals increasing from 10 to 60 minutes. TNF-α mRNA levels were determined by semi-quantitative RT-PCR. **B** Results from 3 experiments are expressed graphically. Data are calculated from ratios of TNF-α mRNA levels over actin mRNA levels. * $p < 0.05$ vs. time 0, ** $p < 0.01$ vs. time 0, and *** $p < 0.001$ vs. time 0

Using the data obtained from mRNA stability experiments, we calculated the mRNA half-life for each experiment and compared TNF-α mRNA half-life from PMA/PHA-stimulated cells with that from cells treated with PMA/PHA in combination with CGS 21680. TNF-α mRNA half-life in PMA/PHA-stimulated U937 cells was 80 ± 12 minutes whereas CGS 21680 pre-treatment decreased significantly ($p < 0.05$) mRNA half-life to 37 ± 8 minutes (Figure 2.10). These data suggest that CGS 21680 decreases both stability and half-life of TNF-α mRNA.

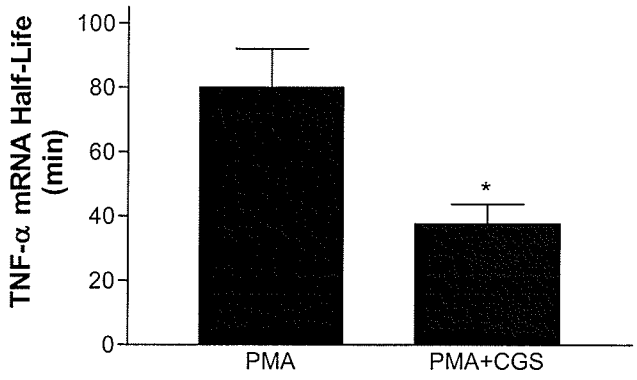

Figure 2.10: Adenosine A_{2A} receptor activation decreases TNF-α mRNA half-life. Half-life of TNF-α mRNA was calculated from experiments performed in Figure 2.8 to determine mRNA stability and statistics were determined using a two-tailed t test. * $p < 0.05$ agonist v. PMA/PHA

Discussion

Adenosine and adenosine receptor agonists have anti-inflammatory and immune modulatory effects primarily by influencing the production of cytokines. By blocking the production of the pro-inflammatory cytokine TNF-α, adenosine receptors can help regulate the activation and function of phagocytes and T cells by shifting immune responses from being cell-mediated to being humoral mediated responses. Because regulation of immune responses through an endogenous system like adenosine may play an important role in the normal function of the immune system and development of immune-related disorders, elucidation of the mechanisms by which TNF-α production is controlled is important for understanding how the immune system controls infection and may contribute to disease.

Production of TNF-α induced by inflammatory stimuli in monocytes and macrophages involves activation of transcription factors, which can bind to the TNF-α promoter and initiate transcription (Dumitru et al., 2000; Ghosh and Karin, 2002; Lee et al., 2000; Liu et al., 2000; Paludan et al., 2001; Raabe et al., 1998; Rao, 2001; Shi et al., 2002; Tsai et al., 2000; Tsai et al., 1996; Udalova and Kwiatkowski, 2001; Zhu et al., 2000). Our experiments were designed to identify transcription factors involved in PMA/PHA-induced TNF-α production that may be targets of adenosine A_{2A} receptors and may be responsible for the anti-TNF-α effects of these receptors. We showed that NF-κB activity is important for PMA/PHA-induced production of TNF-α in U937 cells because pre-treatment of

cell cultures with the NF-κB peptide inhibitor SN50 inhibited TNF-α protein production at a concentration shown to inhibit NF-κB activity (Lin et al., 1995), whereas the inactive SN50 inhibitor peptide had no significant effect of TNF-α protein production. Although NF-κB binding activity was increased over basal levels by PMA/PHA, pre-treatment with CGS 21680 did not block this effect, suggesting that NF-κB binding activity is not affected by adenosine A_{2A} receptor activation.

Other groups studying adenosine-mediated control of cytokine production have shown contradictory results with respect to the affect of adenosine and adenosine receptor agonists on NF-κB. Specifically, studies using RAW 264.1 macrophages and PMA-differentiated U937 macrophages showed no effect of CGS 21680 or the A_1/A_3 receptor agonist I-ABA on the binding activity and nuclear translocation of NF-κB (Hasko et al., 1996; Sajjadi et al., 1996). On the other hand, in THP-1 cells CGS 21680 decreased NF-κB driven transcription but not binding activity (Bshesh et al., 2002). Because both studies used lipopolysaccharide as the inflammatory stimulus the only obvious difference between the studies was the cell type used. Although activation of immune cells by LPS involves CD14 and TollR4 (Cohen, 2002; Dobrovolskaia and Vogel, 2002) the signaling pathways involved may differ between cell types. As a result, NF-κB binding activity activated by LPS in macrophage cell lines (RAW 264.1 and PMA-differentiated U937s) may involve different signaling mechanisms than in undifferentiated monocyte cell lines (THP-1) and may account for differing

effects of CGS 21680 on NF-κB in monocytes and macrophages. Although we used undifferentiated U937 cells, which are pro-monocytic, the effect of adenosine A_{2A} receptors on NF-κB binding activity in our model is consistent with what others have seen in differentiated macrophages, suggesting a similar signaling pathway targeting NF-κB and TNF-α.

Activation of TNF-α gene expression involves other transcription factors in addition to NF-κB. The TNF-α promoter contains binding sites for ATF-2 and cJun, transcription factors that others have implicated in TNF-α production. Our data shows that PMA/PHA increased TNF-α production without increasing basal phosphorylation levels of either ATF-2 or cJun, indicating that PMA/PHA did not increase the binding activity of these transcription factors. Consequently, we did not conduct experiments to test whether activation and phosphorylation of these transcription factors was blocked by CGS 21680.

Because CGS 21680 did not decrease PMA/PHA-induced increases in NF-κB binding activity and PMA/PHA did not increase phosphorylation of ATF-2 and cJun, we designed experiments to determine if adenosine A_{2A} receptor activation blocked PMA/PHA-induced increases in TNF-α gene expression. We showed that CGS 21680 activation of A_{2A} receptors decreased PMA/PHA-induced increases in TNF-*α* protein levels without affecting PMA/PHA-induced increases in steady-state TNF-*α* mRNA levels. In addition, CGS 21680 added simultaneously with, or 30 minutes or 1 hour after PMA/PHA-stimulation had anti-

TNF-α effects similar to those seen when cells were pre-treated with CGS 21680 for 30 minutes. Because pre-treatment of U937 cells with CGS 21680 did not block PMA/PHA induced TNF-α gene expression and because the anti-TNF-α effect of CGS 21680 was similar if the drug was added before, or up to 1 hour after PMA/PHA stimulation, time points which showed significant increases in TNF-α mRNA levels but undetectable TNF-α protein levels, regulation of TNF-α by CGS 21680 is likely a post-transcriptional event and not a consequence of blocking transcription factor activation or initiation of TNF-α gene expression.

Several groups have investigated the effects of adenosine and adenosine receptor agonists on TNF-α mRNA and protein levels. Consistent with our data, adenosine inhibited TNF-α protein production induced by LPS if added before, simultaneously with, or after LPS stimulation in human monocytes (Le Moine et al., 1996), in the monocyte cell line THP-1 (Bshesh et al., 2002), and in neonatal rat myocytes (Wagner et al., 1998). The A_2 receptor agonist DPMA also inhibited TNF-α production when added before, simultaneously with, or after LPS in rat neonatal mycocytes (Wagner et al., 1998).

Taken together, studies investigating the regulation of TNF-α mRNA by adenosine and adenosine receptor agonists suggests that different mechanisms of regulation may exist and may depend on the differentiation state of the cell type used. Some studies have shown decreased LPS-induced steady-state TNF-α mRNA levels induced by the adenosine receptor agonist 2-

chloroadenosine in RAW 264.1 macrophages (Firestein et al., 1994) and in PMA-differentiated U937 cells (Sajjadi et al., 1996). Adenosine and the A_2 receptor agonist DPMA both decreased TNF-α mRNA levels in cardiomyocytes and failing human heart (Wagner et al., 1999). The studies showing decreased TNF-α mRNA by adenosine were conducted in differentiated macrophages, whereas studies showing that adenosine did not block TNF-α gene transcription were conducted in pro-monocytic or monocytic cell lines, suggesting differences in regulation between differentiated and undifferentiated cells. Of significance are recent findings by McKenzie et al, who showed that although TNF-α mRNA abundance and TNF-α/GAPDH mRNA ratios were similar during differentiation of monocytes to macrophages following LPS stimulation, TNF-α protein secretion increased during differentiation and TNF-α mRNA stability increased from 20-30 minutes to 60 minutes after monocytes were differentiated for 5 days (MacKenzie et al., 2002). These differences suggest possible changes in post-transcriptional control of TNF-α mRNA between monocytes and mature macrophages and may contribute to differences seen in adenosine regulation of TNF-α mRNA in monocytes and macrophages. Taken together, our data and data from other groups suggest that the cellular mechanisms regulating the TNF-α mRNA effects of adenosine are receptor and cell-type specific and may be different in monocytes and differentiated macrophages.

TNF-α protein production is controlled post-transcriptionally by mRNA half-life, translation and processing (Baseggio et al., 2002; Clark, 2000; Han et al., 1991;

MacKenzie et al., 2002). As a result, we tested the hypothesis that activation of adenosine A_{2A} receptors blocks TNF-α protein production by decreasing mRNA stability and half-life. We found that pre-treatment of U937 cells with CGS 21680 decreased TNF-α mRNA stability and half-life from 80 minutes with PMA/PHA alone to 37 minutes in combination with CGS 21680. By decreasing mRNA stability and mRNA half-life, CGS 21680 can block TNF-α protein production induced by PMA/PHA without affecting initiation of transcription of the TNF-α gene or of steady-state mRNA levels.

Proteins whose expression is transient, particularly cytokines and chemokines, have mRNA with short half-lives that are less stable than mRNA encoding proteins like GAPDH. mRNA encoding transiently expressed proteins like TNF-α contain adenosine/uridine-rich elements (AREs) in the 3' untranslated region (UTR) that flank the protein coding sequence (Clark, 2000; MacKenzie et al., 2002). mRNAs encoding IL-1α, IL-1β, IL-6, IL-8, IFN-γ, and GM-CSF also contain AREs in the 3' UTR (Kishore et al., 1999). AREs contain one or multiple copies of the sequence AUUUA and destabilize mRNA, shortening its half-life (Clark, 2000). Mammalian TNF-α mRNA contains a well-conserved ARE in the 3'UTR which contains 7 or 8 copies of the AUUUA sequence leading to an average half-life of approximately 30 minutes in human monocytes (Clark, 2000; MacKenzie et al., 2002). Kollias and collegues (Kontoyiannis et al., 1999) developed a mouse with the ARE deleted from the TNF-α gene. LPS stimulation resulted in larger, slower and more sustained induction of TNF-α mRNA as a

consequence of no ARE. TNF-α mRNA half-life controlled through AREs can be influenced by inflammatory stimuli. LPS increased TNF-α mRNA stability and half-life, promoting TNF-α protein production (Clark, 2000; MacKenzie et al., 2002). Our data show that PMA/PHA-induced TNF-α mRNA has a half-life of 80 minutes, longer than 30 minutes observed in human monocytes, therefore like LPS, PMA/PHA may increase mRNA stability as a means of promoting TNF-α protein production. Because activation of adenosine A_{2A} receptors decreased TNF-α mRNA stability and half-life, this regulation by CGS 21680 may be mediated by the 3'UTR AREs possibly by altering a cell signaling pathway that normally controls ARE function and mRNA stability.

Regulation of TNF-α by adenosine appears to use cell-type and stimulus specific signaling mechanisms with significant differences observed between monocytes and macrophages with respect to actions on transcription factor activity and induction of mRNA levels. These differences suggest that adenosine may use different regulatory mechanisms to control cytokine production depending on the differentiation state of cells of the monocytic lineage. Our studies using pro-monocytic U937 cells show that CGS 21680 regulated TNF-α post-transcriptionally at least partially by decreasing mRNA stability and half-life. Similar to results observed with LPS, PMA/PHA increased TNF-α mRNA stability from 20-30 minutes in human monocytes to 80 minutes in U937 cells, identical to the half-life seen in LPS-stimulated differentiated macrophages. Our data with U937 cells are also similar to data from macrophages showing no effect of

adenosine A_{2A} receptors on NF-κB binding activity, suggesting that the anti-TNF-α actions of adenosine in U937 cells resemble the anti-TNF-α mechanisms observed by others in macrophages rather than in monocytes.

In summary we propose that adenosine A_{2A} receptors mediate TNF-α protein production by decreasing TNF-α mRNA stability and half-life, and increasing mRNA degradation. TNF-α mRNA stability is controlled by AREs in the 3'UTR, a mechanism that is conserved for mRNA encoding other transiently expressed proteins, particularly those involved in immune responses. As a result, through this regulatory mechanism, adenosine A_{2A} receptors may efficiently control the expression of several immune proteins by decreasing the stability of mRNA in the same manner. Our findings are important because they strengthen the possibility of targeting the adenosine receptor system therapeutically for immune and inflammatory disorders. Regulation of immune proteins post-transcriptionally, without the requirement for drug pre-treatment, lengthens the therapeutic window for pharmaceuticals targeted at adenosine receptors. In addition, regulation of protein expression through a mechanism that targets transiently expressed proteins like those activated during an immune response may be a useful property that minimizes side effects.

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Chapter 3

Role of Protein Phosphatase and p38 Activity in Adenosine A_{2A} Receptor Regulation of TNF-α Production

Abstract

Activation of mitogen-activated protein kinase (MAPK) signaling pathways induces TNF-α gene expression and protein production in macrophages. JNK, ERK, and p38 are three members of the MAPK signaling family that have been shown to regulate TNF-α production and do so by increasing transcription factor binding activity and by regulating translation and stability of TNF-α mRNA. Normally, the MAPK signaling pathways that are involved in pro-inflammatory cytokine production are activated when phosphorylated by kinases and inactivated when dephosphorylated by phosphatases. We hypothesized that adenosine A_{2A} receptor activation inhibits TNF-α production by decreasing MAPK signaling activity through increased phosphatase activity. First, we determined that although adenosine A_{2A} receptors are coupled to adenylyl cyclase and cAMP, and cAMP-dependent signaling pathways generally mediate actions of these receptors, the anti-TNF-α effects of the A_{2A} receptor agonist CGS 21680 were independent of cAMP. Next, we examined the effects of CGS 21680 on MAPK signaling. PMA/PHA-induced increases in TNF-α production required ERK, p38, and JNK activity, but only ERK phosphorylation was increased by PMA/PHA. A_{2A} receptor activation did not decrease phospho-ERK levels or basal phospho-JNK levels but decreased basal phospho-p38 levels. In addition, the anti-TNF-α effects of CGS 21680 were mediated by serine/threonine phosphatase activity because okadaic acid significantly blocked CGS 21680-mediated inhibition of TNF-α production and CGS 21680 increased cytosolic serine/threonine phosphatase activity in U937 cells. These data suggest that

adenosine A_{2A} receptors control TNF-α production by activating a serine/threonine phosphatase and by blocking p38, a signaling pathway involved in stabilizing TNF-α mRNA.

Introduction

Functionally, serine/threonine phosphatases have been implicated in regulating signaling pathways involved in inflammation and cytokine production. Protein phosphatase 2A (PP2A) can regulate the activity of IκBα kinase, which activates NF-κB binding activity by phosphorylating the NF-κB inhibitor protein IκBα and pharmacological inhibition of PP2A can increase NF-κB binding activity (Shanley et al., 2001). PP2A can also regulate the JNK signaling pathway in monocytes, ultimately altering the expression of the pro-inflammatory cytokine IL-1β (Shanley et al., 2001).

Signals from membrane-bound receptors are transmitted within a cell to the nucleus by a complex network of proteins whose activity is tightly regulated. Many cell signaling pathways are controlled by phosphorylation and the efficiency of these signaling cascades results from the balance between kinase and phosphatase activities (Tamura et al., 2002; Zhang et al., 2002). The human genome encodes approximately 500 kinases, of which two-thirds phosphorylate serine and threonine residues and one-third phosphorylate tyrosine residues. Of the approximately 150 protein phosphatases less than 40 are selective for serine and threonine residues (Cohen, 2002). The seemingly mismatched number of serine/threonine kinases and phosphatases suggests that serine/threonine phosphatases may have broad specificity or alternative means of regulating substrate specificity. Indeed, protein phosphatase 1 (PP1) for example signals through one of five catalytic subunits (PP1c) that can complex with any of 50

regulatory subunits. The regulatory subunits can direct substrate specificity, subcellular localization, and regulate phosphatase activity (Cohen, 2002). Other serine/threonine phosphatases like protein phosphatase 2A (PP2A) are regulated in a similar manner by regulatory subunits and signal through catalytic subunits. Substrate specificity and functional diversity of serine/threonine phosphatases result primarily from interactions between catalytic subunits and regulatory subunits.

MAPKs have been extensively studied in immune cells because of their essential role in controlling cytokine production. By driving cytokine production, MAPK signaling cascades are pivotal regulators of immune responses. Indeed, MAPKs are particularly important for regulating TNF-α production by activating transcription factor binding activity, RNA polymerase II holoenzyme assembly, translation, and mRNA stability (Zhu et al., 2000). MAPKs are activated by inflammatory or stress stimuli like lipopolysaccharide, which signals through CD14 and Toll-like receptor 4 to activate ERK, JNK, and p38 in macrophages (Means et al., 2000; Zhu et al., 2000). JNK is a stress-activated kinase signaling molecule and a member of the MAPK family of signaling molecules. JNK is a serine/threonine kinase whose activity is controlled by phosphorylation and is activated when phosphorylated on a threonine residue (Shanley et al., 2001; Tamura et al., 2002). ERK and p38 are also members of the MAPK signaling family and are implicated in regulating stress responses of a cell. MAPK signaling cascades control physiological functions in addition to stress responses

like protein production, neurotransmitter receptor function, and cell cycle progression. MAPK signaling cascades phosphorylate proteins like cJun, ATF-2, and IκBα kinase, which activate transcription factors, that bind to *cis* elements like Egr-1, CRE, κB, AP-1 on the TNF-α promoter (Dumitru et al., 2000; Falvo et al., 2000; Fuchs et al., 2000; Liu et al., 2000; Rao, 2001; Shi et al., 2002; Tsai et al., 2000; Tsai et al., 1996; Udalova and Kwiatkowski, 2001; Zhu et al., 2000). MAPKs also regulate protein expression through activation of proteins involved in translation like eIF4E and 4EBP-1 (Liu et al., 2002; Potter et al., 2001; Raught and Gingras, 1999; Rolli-Derkinderen et al., 2003; Wang et al., 1998). Finally, regulation of mRNA stability by MAPK has been most extensively studied for p38. p38 activation results in stabilization of mRNA encoding transiently expressed proteins, an effect that is particularly important for regulation of TNF-α in monocytes and macrophages (Baseggio et al., 2002; Brook et al., 2000; Chen and Shyu, 1995; Crawford et al., 1997; Han et al., 1991; Kishore et al., 2001; Kishore et al., 1999; Mahtani et al., 2001; Manthey et al., 1998; Rutault et al., 2001; Wang et al., 1999). JNK has also been implicated in regulation of mRNA stability through a similar mechanism as p38 specifically for IL-3 in mast cells (Ming et al., 1998). A simplified schematic outlining the activation and biological functions of the major MAPK signaling pathways is shown in Figure 3.1.

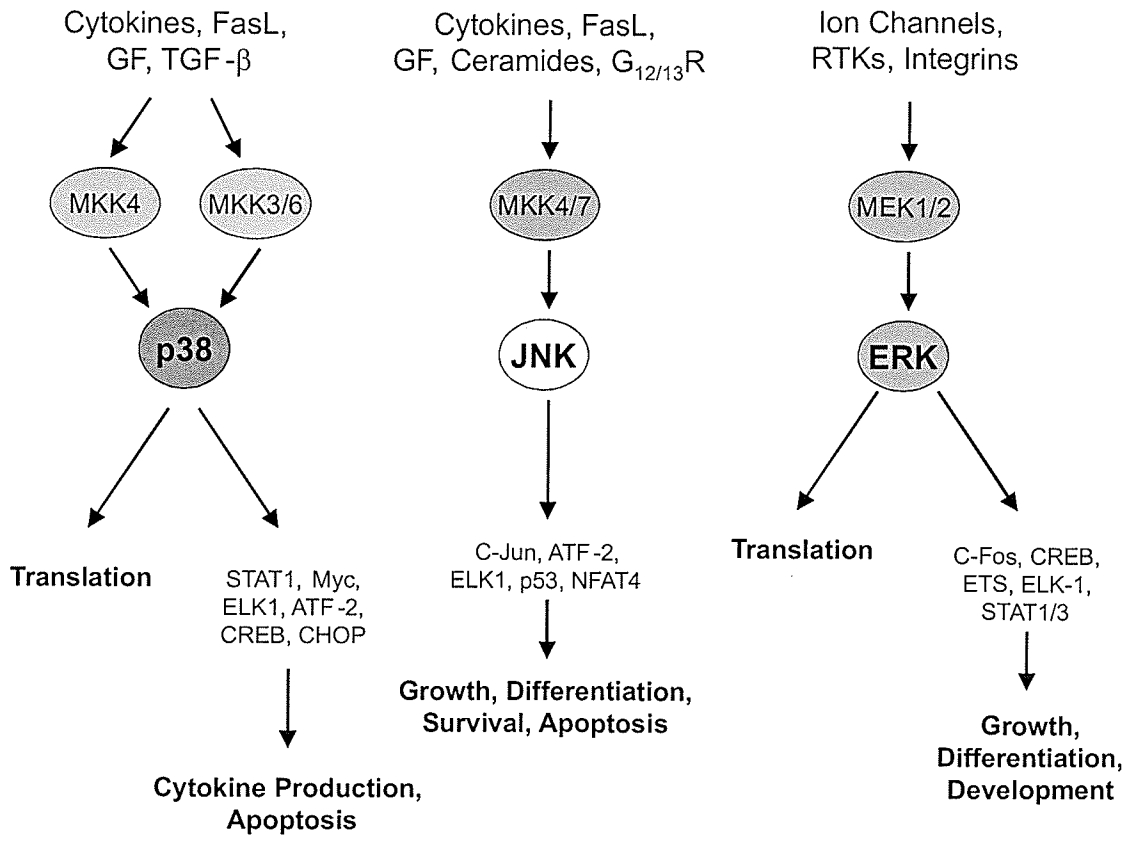

Figure 3.1: Activation and Biological Function of MAPK Cell Signaling Pathways. GF – growth factors, RTK – receptor tyrosine kinase, TGF – transforming growth factor. STAT1, ELK1, ATF-2, CREB, CHOP, cJun, cFos, NFAT, and ETS are all transcription factors.

The diversity of cellular functions activated and controlled by cell signaling cascades like MAPK emphasizes the need for exquisite regulation of their activity. Protein expression, tissue distribution, cellular localization, and interaction with other regulatory proteins all contribute to the variety of functions mediated by 500 kinases (Cohen, 2002). Perhaps more impressive, is that protein phosphatases not only control phosphorylation and activity of kinases, but also of their target substrates. Protein phosphatases can only dephosphorylate their substrates when they are within close vicinity of their targets. As a result,

several phosphatases physically bind to their kinase substrates to promote tight regulation of activity and substrate specificity. For example, ERK-2 can bind to the MAPK phosphatase MKP-3 (Shanley et al., 2001). Although MAPK signaling cascades have been extensively mapped out in immune cells in particular, less is known about regulation and contribution of phosphatases to immune responses. Understanding of the complex signaling pathways regulating immune responses and cytokine production must involve the study of both kinases and phosphatases as both may prove to be attractive targets for pharmaceutical intervention. To emphasize this possibility is recent work showing the ability of double stranded RNA viruses to shut down protection mediated by interferon. Normally, the immune response to infection with viruses like herpes simplex virus is to upregulate production of interferon, a cytokine that activates a protein kinase that shuts down protein synthesis in the host cell and prevents replication of the virus. Herpes simplex virus produces a protein which complexes with and activates PP1c, turning off this protective response (Cohen, 2002). In addition, the immunosuppressive drugs cyclosporin and FK506 target the catalytic site of PP2B and block its activity. PP2B normally functions in immune cells by dephosphorylating and activating the transcription factor NFAT (Shanley et al., 2001). NFAT is one of few examples of proteins that are active in a dephosphorylated state and blocking dephosphorylation of NFAT decreases its transcriptional activity and dampens immune responses (Tsai et al., 1996).

To elucidate the signaling mechanisms mediating the immune regulatory effects of adenosine receptors, experiments in this chapter were designed to identify

kinase and phosphatase signaling pathways involved in TNF-α production and regulation by adenosine A_{2A} receptors.

Methods

Pharmacology: To determine if the anti-TNF-α effect of CGS 21680 was dependent on the cAMP/PKA signaling pathway, U937 cells were pre-treated with H89, a PKA inhibitor, or Rp-8-Br-cAMPS, a cAMP antagonist, for 15 minutes prior to activation of A_{2A} receptors with CGS 21680. Following a 30 minute pre-treatment of U937 cells with CGS 21680, cell cultures were stimulated with PMA/PHA for 4 hours, supernatants were collected, and TNF-α levels were determined by ELISA as described in the Appendix.

To identify the signaling pathways activated by PMA/PHA required for TNF-α production, U937 cells were pretreated for 15 minutes with PD 98059 to inhibit ERK, SB 202190 to inhibit p38, SP 600125 to inhibit JNK, or wortmannin to inhibit PI3K. U937 cell cultures were then stimulated for 4 hours with PMA/PHA, supernatants were collected and TNF-α levels were determined by ELISA as described in the Appendix.

To determine if the anti-TNF-α effect of CGS 21680 was mediated by a protein phosphatase, U937 cells were pre-treated with okadaic acid to inhibit serine/threonine phosphatases PP1 and PP2A, dephostatin to inhibit tyrosine phosphatases, or sodium orthovanadate to inhibit tyrosine phosphatases for 15 minutes prior to A_{2A} receptor activation. Following treatment of U937 cells with CGS 21680 for 30 minutes, cell cultures were stimulated for 4 hours with

PMA/PHA, supernatants were collected and TNF-α levels were determined by ELISA as described in the Appendix.

Serine/Threonine Phosphatase Assays: For time course experiments, U937 cells were treated with CGS 21680 for time intervals ranging from 5 to 60 minutes, cell pellets were collected on ice, cytosol and membrane subcellular fractions were isolated, and serine/threonine phosphatase activity was measured using the commercially available enzyme assay kit (New England Biolabs). Both protocols are described in detail in the Appendix.

For dose response experiments, U937 cells were treated with CSG 21680 at varying concentrations for 10 minutes and serine/threonine phosphatase activity was measured in approximately 100 ng of cytosolic protein fractions.

For pharmacological experiments, U937 cells were pre-treated with ZM 241385 for 5 minutes or with okadaic acid for 15 minutes prior to activation of A_{2A} receptors with CGS 21680. Following A_{2A} receptor activation for 10 minutes, serine/threonine phosphatase activity was measured in approximately 100 ng of cytosolic protein extract. For experiments using the specific PP1 peptide inhibitor, serine/threonine phosphatase enzyme assay was conducted in the presence of PPI2 with approximately 100 ng of cytosolic protein extract isolated from cells treated with CGS 21680 for 10 minutes.

Western Blots: The protocols used for western blots are outlined in detail in the Appendix. Briefly, whole cell lysates were separated on 8-12% SDS-PAGE gels run at 80V for approximately 15 minutes, then 100V for approximately 1.5-2 hours. Proteins were transferred from the gels onto nitrocellulose membranes using a semi-dry transfer apparatus at 15V for 30 minutes or overnight using a wet transfer apparatus at 35V. All membranes were blocked with 1% bovine serum albumin (BSA) prepared in 1XTBS at room temperature for 3 hours or overnight at $4^{O}C$. Primary antibodies were diluted in 0.5% BSA (anti-p-ERK, p-p38, p38, p-JNK, p-eIF4E, p-4EBP1, and actin antibodies were diluted 1:200) prepared in 1 X TBS and incubated with nitrocellulose membranes overnight at $4^{O}C$. Membranes were developed using HRP-conjugated secondary antibodies and ChemiGlow ECL substrate kit (Alpha Innotech). Bands were visualized using a FluorS Max Imaging System (BioRad Laboratories).

RT-PCR: U937 cells were pre-treated with SB 202190 for 15 minutes prior to stimulation with PMA/PHA. Cell cultures were stimulated for 2 or 4 hours, cell pellets were isolated, total RNA was extracted, cDNA was synthesized, and TNF-α mRNA levels were determined by RT-PCR. Supernatants from these same cells were collected for determination of TNF-α levels by ELISA.

Results

Adenosine A_{2A} receptor activation blocks TNF-α production in a cAMP-independent manner. Adenosine A_{2A} receptors are G-protein receptors coupled to adenylyl cyclase and most physiological effects of this receptor result from activation of adenylyl cyclase and production of cAMP (Fredholm et al., 2001). Once produced, cAMP activates protein kinase A (PKA) and PKA-dependent signaling pathways. Adenosine has been shown to regulate cytokine production in a manner dependent on cAMP and PKA (Bshesh et al., 2002; Wagner et al., 1998). As a result, we hypothesized that the anti-TNF-α effects of A_{2A} receptors would depend on production of cAMP and activation of PKA. To test this hypothesis, we pre-treated U937 cells for 15 minutes with the PKA inhibitor H89 or the cAMP antagonist Rp-8-Br-cAMPS prior to activation of A_{2A} receptors with CGS 21680. Neither H89 nor Rp-8-Br-cAMPS altered the anti-TNF-α effect of CGS 21680, suggesting that this effect is independent of cAMP production and PKA activation (Figure 3.2). To ensure that H89 and Rp-8-Br-cAMPS had the desired effects, both drugs reversed the anti-TNF-α effect of the adenylyl cyclase activator forskolin suggesting that the desired pathways were inhibited (data not shown).

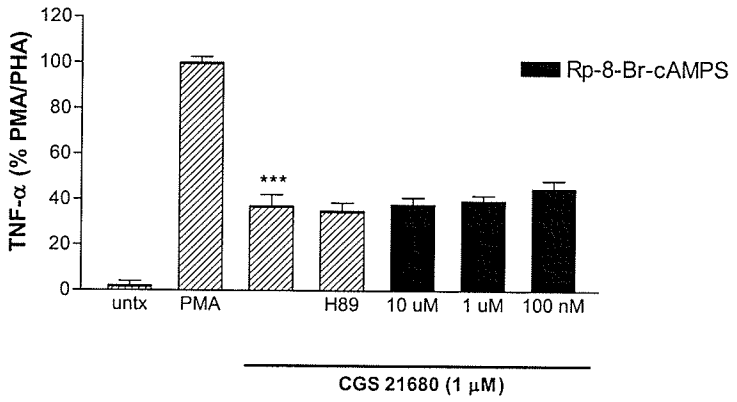

Figure 3.2: Adenosine A_{2A} receptor-induced inhibition of TNF-α is independent of cAMP and PKA. U937 cells were untreated (untx) or were treated with CGS 21680 (1 μM) in combination with H89 (100 nM) or Rp-8-Br-cAMPS at concentrations of 10, 1 or 0.1 μM prior to stimulation with PMA/PHA. After 4 hours, supernatants were collected and TNF-α levels were determined by ELISA. *** $p < 0.001$ vs. PMA/PHA

PMA/PHA-induced TNF-α production requires MAPK signaling pathways. Production of TNF-α in macrophages by inflammatory stimuli requires MAPK signaling pathways including ERK, JNK, and p38 (Zhu et al., 2000). Furthermore, studies from our group have shown that TNF-α production in macrophages induced by the HIV-1 protein Tat required phospholipase C (unpublished data). Accordingly, we designed experiments to investigate the specific kinase signaling pathways involved in PMA/PHA-induced TNF-α protein production. We treated U937 cells with PD 98059, an ERK inhibitor, SB 202190, an inhibitor of p38, SP 600125, an inhibitor of JNK, wortmannin, an inhibitor of PI3K, or U73122, an inhibitor of phospholipase C for 15 minutes prior to A_{2A} receptor activation. All kinase inhibitors tested inhibited TNF-α protein production to varying degrees (Figure 3.3). PD 98059 inhibited TNF-α levels by 83 ± 5% ($p < 0.001$), SB 202190 inhibited TNF-α levels by 79 ± 7% ($p < 0.001$),

SP 600125 inhibited TNF-α levels by 71 ± 2% ($p < 0.001$), and wortmannin inhibited TNF-α levels by 44 ± 9% ($p < 0.001$). In addition, U73122 dose-dependently inhibited TNF-α levels with maximum inhibition of 92 ± 3% ($p < 0.001$) at 10 μM. These data suggest that ERK, p38, JNK, PI3K, and PLC signaling pathways are all involved in regulating PMA/PHA-induced TNF-α production in these cells.

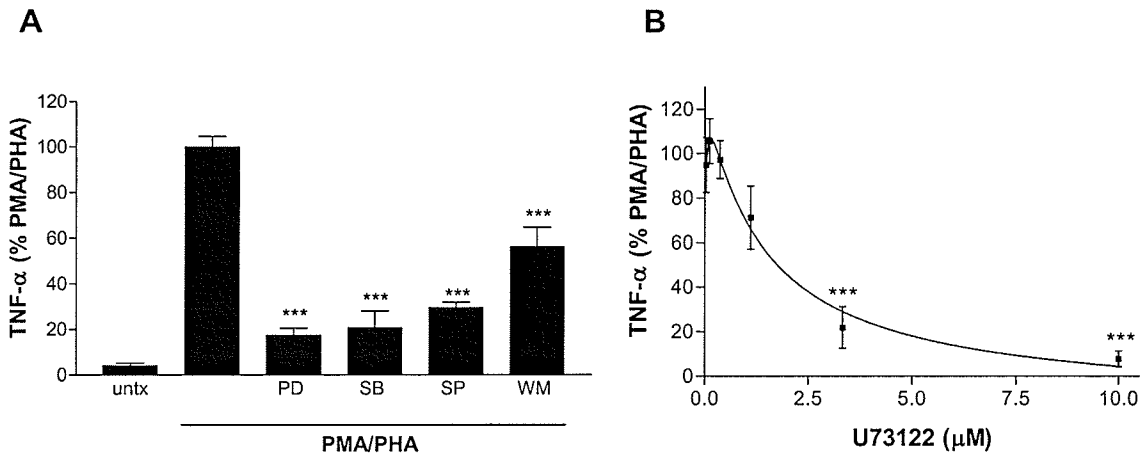

Figure 3.3: PMA/PHA-induced TNF-α production requires MAPK and PLC signaling pathways. **A** U937 cells were untreated (untx) or were treated with PD 98059 (1 μM) an inhibitor of ERK, SB 202190 (1 μM) an inhibitor of p38, SP 600125 (10 μM) an inhibitor of JNK, or wortmannin (100 nM) an inhibitor of PI3K, for 15 minutes prior to PMA/PHA stimulation. **B** U937 cells were treated with U73122, an inhibitor of PLC, for 15 minutes prior to PMA/PHA stimulation. For both figures, cells were stimulated for 4 hours, supernatants were collected and TNF-α levels were determined by ELISA. *** $p < 0.001$ vs. PMA/PHA

Adenosine A_{2A} receptor activation Inhibited TNF-α production in a protein phosphatase-dependent manner. PMA/PHA-induced TNF-α production involves activation of several cell signaling pathways including ERK, p38, and JNK and these kinase signaling pathways are regulated by phosphorylation (Tamura et al.,

2002). For each MAPK signaling family member, the active protein is phosphorylated on serine, threonine, and/or tyrosine residues. We hypothesized that activation of adenosine A_{2A} receptors could block TNF-α by activating a protein phosphatase and thereby dephosphorylate active MAPK signaling family members and decrease activity of MAPK signaling pathways. To test this hypothesis, we treated U937 cells for 15 minutes with okadaic acid to block PP1 and PP2A serine/threonine phosphatases, dephostatin to block tyrosine phosphatases, or sodium orthovanadate to block tyrosine phosphatases prior to A_{2A} receptor activation. Cell cultures were then stimulated with PMA/PHA and TNF-α protein levels determined by ELISA. Pre-treatment of U937 cells with 100 nM okadaic acid resulted in a statistically significant ($p < 0.05$) inhibition of the anti-TNF-α effects of CGS 21680 whereas lower concentrations of okadaic acid had no effect (Figure 3.4A). Because okadaic acid at 100 nM blocks both PP1 and PP2A whereas lower concentrations block only PP2A, these data suggest that the anti-TNF-α effect of CGS 21680 may involve activation of PP1. Dephostatin and sodium orthovanadate (100 μM) did not reverse the anti-TNF-α effects of CGS 21680. However, pre-treatment with 10 μM sodium orthovanadate partially reversed the anti-TNF-α effects of CGS 21680 (Figure 3.4B). These results are confusing and counterintuitive because only the lower dose of sodium orthovanadate reversed the anti-TNF-α effects of CGS 21680. Our initial experiments with tyrosine phosphatase inhibitors were performed with sodium orthovanadate which is a broad spectrum inhibitor that also inhibits Na^+K^+ ATPase, acid and alkaline phosphatases, phosphofructokinase, and

adenylate kinase. We went on to test the effects of dephostatin, a more specific tyrosine phosphatase inhibitor and our results suggested that tyrosine phosphatases were not a predominant pathway mediating the anti-TNF-α effects of CGS 21680. The effects of sodium orthovanadate may result from actions of this drug on several cell signaling pathways.

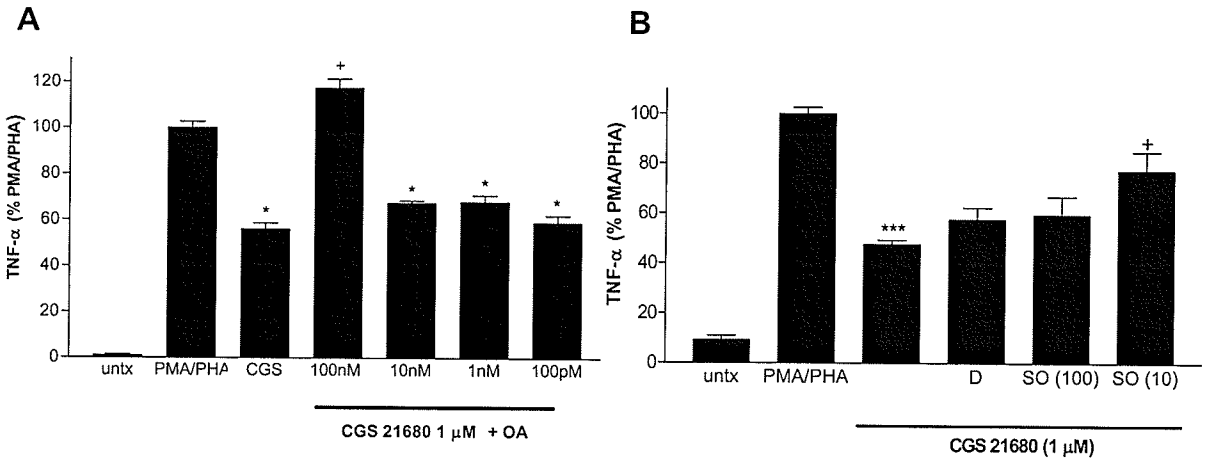

Figure 3.4: Adenosine A_{2A} receptor activation inhibits TNF-α production by activating a protein phosphatase. **A** U937 cells were untreated (untx) or were treated with okadaic acid (OA) to inhibit serine/threonine protein phosphatases PP1 and PP2A, for 15 minutes prior to A_{2A} receptor activation with CGS 21680 (1 μM). **B** U937 cells were treated with sodium orthovanadate (SO, μM) or dephostatin (D, 1 μM), both tyrosine phosphatase inhibitors, for 15 minutes prior to A_{2A} receptor activation with CGS 21680 (1 μM). For both figures, cells were stimulated for 4 hours, supernatants were collected and TNF-α levels were determined by ELISA. * $p < 0.05$ vs. PMA/PHA, *** $p < 0.001$ vs. PMA/PHA, and + $p < 0.05$ vs. CGS 21680

Adenosine A_{2A} receptor activation increases cytosolic serine/threonine phosphatase activity. Because the anti-TNF-α effects of adenosine A_{2A} receptor activation were reversed following incubation with the PP1/PP2A inhibitor okadaic acid, we hypothesized that activation of adenosine A_{2A} receptors would increase serine/threonine phosphatase activity. We treated U937 cells with CGS

21680 (1 μM) for time intervals ranging from 5 to 60 minutes, isolated membrane and cytosol fractions from each sample, and measured serine/threonine phosphatase activity using a commercially available enzyme assay kit. Compared with untreated cells, CGS 21680 treatment of U937 cells for 10 minutes increased cytosolic serine/threonine phosphatase activity by 27 ± 5% ($p < 0.01$) (Figure 3.5A). The increases in phosphatase activity observed after 10 minutes were decreased by 20 to 30 minutes and were similar to control levels by 60 minutes. In contrast, no significant change in phosphatase activity was detected in membrane fractions of any time point tested following CGS 21680 treatment although a non-statistically significant trend towards an increase in activity was evident when cells were treated with CGS 21680 for up to 60 minutes (Figure 3.5B).

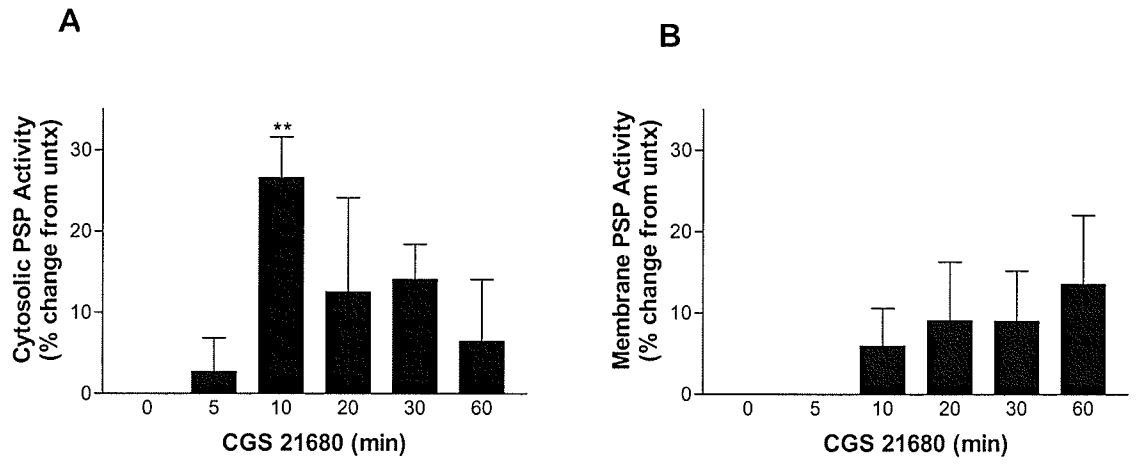

Figure 3.5: Adenosine A_{2A} Receptor Activation Increases Serine/Threonine Phosphatase Activity. U937 cells were untreated (untx) or treated with CGS 21680 (1 μM) for intervals from 5 to 60 minutes. Serine/threonine phosphatase activity was measured using an enzyme assay kit from New England Biolabs in cytosolic fractions (A) and membrane fractions (B). ** $p < 0.01$ vs. untx

To determine the dose dependency with which CGS 21680 activated cytosolic serine/threonine phosphatase activity, we treated U937 cells for 10 minutes with concentrations of CGS 21680 ranging from 10 to 1000 nM and measured serine/threonine phosphatase activity in cytosolic fractions. Statistically significant increases in activity were observed at CGS 21680 concentrations of 100 and 1000 nM, but not at 10 nM (Figure 3.6).

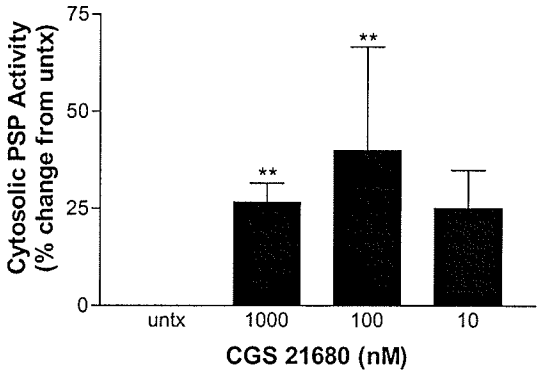

Figure 3.6: Adenosine A_{2A} Receptor Activation Increases Serine/Threonine Phosphatase Activity. U937 cells were untreated (untx) or were treated with CGS 21680 (1 μM) for 10 minutes at concentrations ranging from 10 to 1000 nM. Cytosolic serine/threonine phosphatase activity was measured using an enzyme assay kit from New England Biolabs. ** $p < 0.01$ vs.

We next further examined phosphatase activity induced by CGS 21680 to determine if the CGS 21680-induced increases in enzyme activity would be blocked by pre-treating U937 cells with the A_{2A} receptor antagonist ZM 241385. U937 cells were treated with ZM 241385 at a concentration specific for A_{2A} receptors (Klotz, 2000) for 5 minutes prior to stimulation with CGS 21680 and serine/threonine phosphatase activity was measured in cytosolic fractions after activation of A_{2A} receptors for 10 minutes. Pre-treatment with ZM 241385 did not

block induction of phosphatase activity by CGS 21680 and ZM 241385 alone resulted in a small non-significant increase in phosphatase activity (Figure 3.7). The lack of antagonism of the effect of CGS 21680 by ZM 241385 suggests that the actions of CGS 21680 may be due to other effects including activation of other adenosine receptors. Nevertheless, data illustrated in Figure 3.6 show increased phosphatase activity with CGS 21680 at a concentration as low as 10 nM, known to be specific for A_{2A} receptors (Klotz, 2000).

Recently, it was reported that adenosine A_{2A} receptor agonism activated PP1 in neutrophils and this pathway may contribute to the inhibitory actions of these receptors on neutrophil functions (Bouma et al., 1997; Bullough et al., 1995; Firestein et al., 1995; Revan et al., 1996; Sullivan et al., 1999; Walker et al., 1997). Consequently, we hypothesized that PP1 is activated by CGS 21680 in U937 cells and that the increase in serine/threonine phosphatase activity measured after 10 minutes of stimulation of U937 cells with CGS 21680 is partially a result of PP1 activation. To address this, we pre-treated U937 cells with okadaic acid, a specific inhibitor of PP1 and PP2A, for 15 minutes prior to stimulation with CGS 21680 and measured cytosolic serine/threonine phosphatase activity. Additionally, we measured phosphatase activity in cytosolic fractions isolated from cells treated with CGS 21680 alone or in the presence of a specific PP1 peptide inhibitor, PPI2. Okadaic acid and PPI2 both attenuated CGS 21680-induced increases in phosphatase activity but these changes were not statistically significant; phosphatase activity was decreased

60% by okadaic acid and 27% by PPI2 (Figure 3.7). These data suggest that 60% of serine/threonine phosphatase activity induced by CGS 21680 may be a result of PP1 and PP2A, with 27% being a result of PP1. The remaining serine/threonine phosphatase activity detected following A_{2A} receptor activation likely involves a phosphatase we have not identified with pharmacological inhibitors in these experiments.

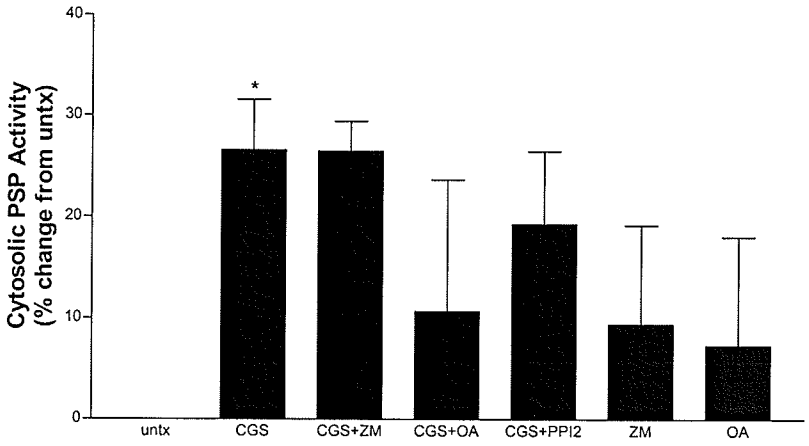

Figure 3.7: Adenosine A_{2A} Receptor Activation Increases PP1 and PP2A Activity. U937 cells were untreated (untx) or were treated with CGS 21680 (1 μM) for 10 minutes in combination with ZM 241385 (100 nM) or with okadaic acid (100 nM) and phosphatase activity was measured in cytosolic fractions. Additionally, enzyme activity was measured in cytosolic fractions from cells treated with CGS 21680 alone or in the presence of PPI2 (10 nM). * $p < 0.05$ vs. untx

Activation of A_{2A} receptors decreases basal levels of phospho-p38. Knowing that three MAPK signaling pathways are involved in PMA/PHA-induced TNF-α production, and that the activity of these pathways is controlled by phosphorylation, we hypothesized that A_{2A} receptors decrease TNF-α production by inhibiting activity (phosphorylation) of the MAPK signaling pathways via

protein phosphatase activation. We have shown that activation of A_{2A} receptors with CGS 21680 increases serine/threonine phosphatase activity and that blocking this phosphatase activity with okadaic acid reverses the anti-TNF-α effect of A_{2A} receptors. We next tested whether activation of A_{2A} receptors decreased activity of the MAPK signaling pathways involved in TNF-α production. To answer this question, we first examined which signaling molecules were activated by PMA/PHA in U937 cells. We treated cells with PMA/PHA for time intervals ranging from 5 to 60 minutes and measured phospho-MAPK levels by western blot with phospho-specific antibodies. PMA/PHA caused statistically significant increases in phospho-ERK levels ($p < 0.05$) for all time points studied (Figure 3.8). In contrast, no statistically significant changes in basal levels of phospho-p38 or phospho-JNK (p54 and p46) were seen with PMA/PHA stimulation (Figure 3.8). These data suggest that in addition to the requirement for ERK in PMA/PHA-induced TNF-α production, treatment of U937 cells with PMA/PHA increases the phosphorylation, and consequently the activity, of both the p42 and p44 isoforms of ERK. Lack of increases in phospho-p38 or phospho-JNK with PMA/PHA was surprising because pharmacological inhibition of both of these signaling pathways significantly decreased PMA/PHA-induced TNF-α levels (Figure 3.3). This suggests alternatively that basal levels of phospho-p38 and phospho-JNK evident in untreated U937 cells (Figure 3.8) may be sufficient for TNF-α production and that blocking this basal level of activity pharmacologically impairs the ability of U937 cells to induce TNF-α production.

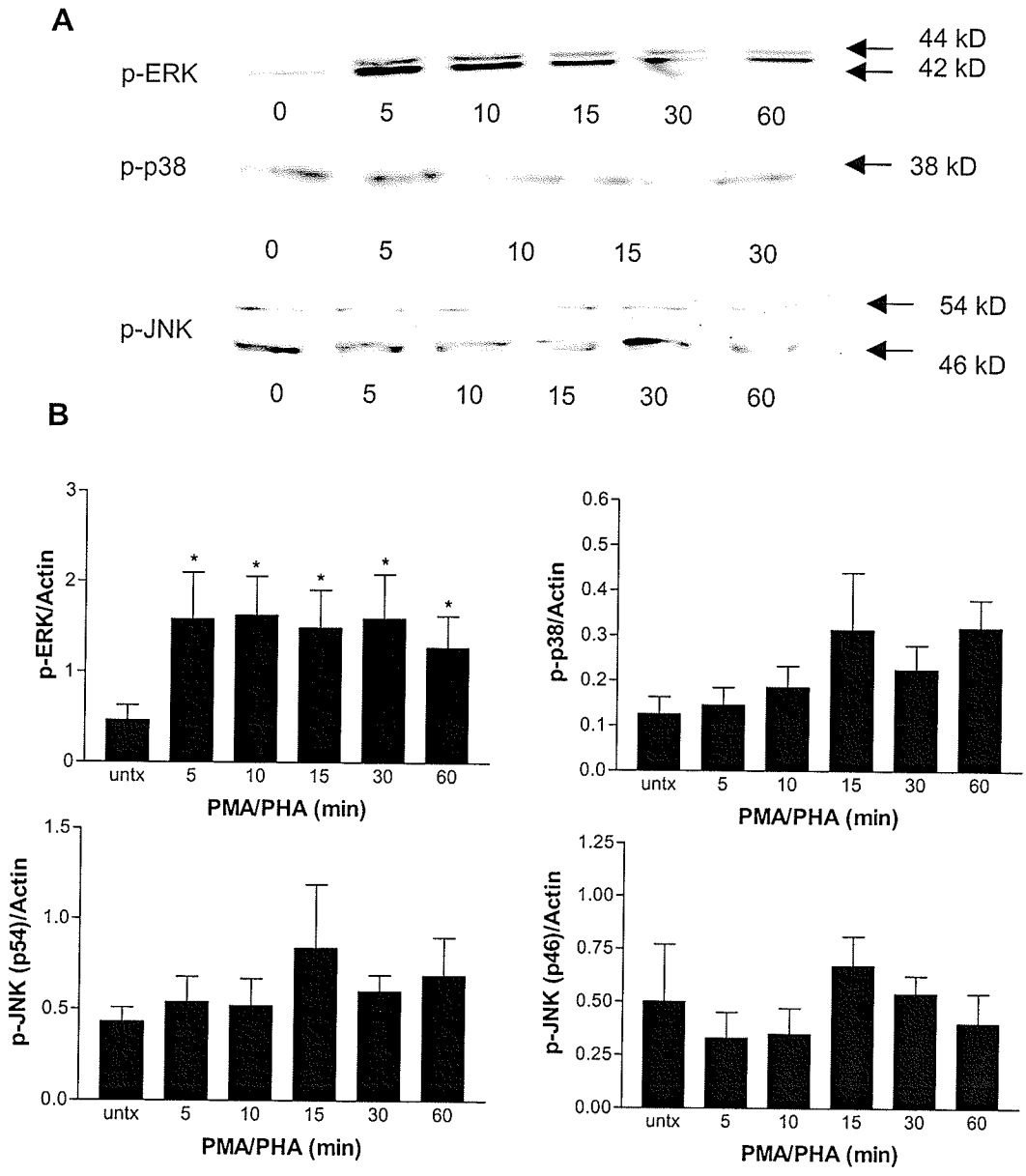

Figure 3.8: PMA/PHA increases protein levels of phospho-ERK but not phospho-p38 or phospho-JNK. **A** U937 cells were untreated (untx) or were treated with PMA/PHA for time intervals increasing from 5 to 60 minutes. Protein levels of phospho-ERK, phospho-p38, and phospho-JNK were determined by western blot with phospho-specific antibodies. **B** Results from 3 independent experiments are shown graphically. * $p < 0.05$ vs. untx

Because PMA/PHA treatment of U937 cells increased phospho-ERK levels and because ERK activity was required for PMA/PHA-induced TNF-α production, we hypothesized that CGS 21680 would decrease PMA/PHA-induced phospho-ERK

protein levels. To test this hypothesis, we treated U937 cells with CGS 21680 prior to PMA/PHA stimulation and phospho-ERK levels were determined by western blot. In addition, we treated U937 cells with the ERK inhibitor PD 98059 alone and in combination with CGS 21680 prior to PMA/PHA stimulation and measured phospho-ERK levels. Pre-treatment of U937 cells with CGS 21680 alone or in combination with ZM 241385 had no effect on PMA/PHA-induced phospho-ERK levels, suggesting that this pathway is not a target of A_{2A} receptor activation as a mechanism for blocking TNF-α production (Figure 3.9). Notably, pre-treatment of U937 cells with the ERK inhibitor PD 98059 alone or in combination with CGS 21680 decreased PMA/PHA-induced phospho-ERK protein levels, verifying that PD 98059 at concentrations used were capable of decreasing activity of the ERK signaling pathway (Figure 3.9).

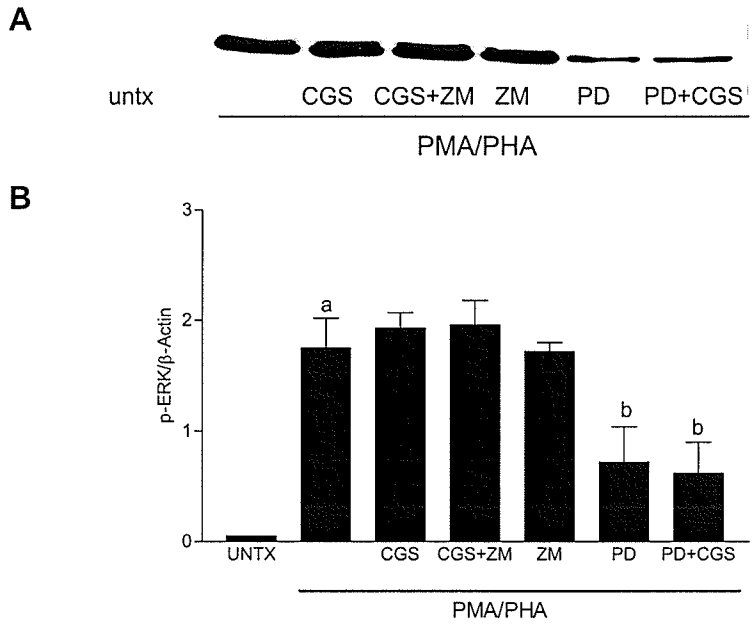

Figure 3.9: Adenosine A_{2A} receptor activation does not decrease PMA/PHA-induced phospho-ERK protein levels. **A** U937 cells were either untreated (untx) or treated with PMA/PHA for 10 minutes in combination with CGS 21680 (1 μM), ZM 241385 (100 nM), or PD 98059 (1 μM). Phospho-ERK levels were determined by western blot with a phospho-specific primary antibody. **B** Results from 4 independent experiments are shown graphically. [a] $p < 0.05$ vs. untx and [b] $p < 0.05$ vs. PMA/PHA

Although each MAPK signaling pathway tested was involved in PMA/PHA-induced TNF-α production, pharmacological inhibition of the ERK and p38 pathways had the most pronounced effect on TNF-α levels, suggesting the importance of these pathways in controlling TNF-α production (Figure 3.3). Although phospho-p38 levels were not increased by PMA/PHA (Figure 3.8), basal levels of phospho-p38 were sufficient to increase production of TNF-α. As such, basal levels of phospho-p38 may provide a target for A_{2A} receptor activation in order to block TNF-α production. As a result, we tested the hypothesis that activation of adenosine A_{2A} receptors would decrease basal levels of phospho-p38 in un-stimulated U937 cells as a mechanism for blocking TNF-α production. U937 cells were treated with CGS 21680 for time intervals ranging from 5 to 30 minutes and phospho-p38 levels were determined by western blot. Both 5 and 10 minute treatments of U937 cells with CGS 21680 resulted in statistically significant ($p < 0.01$ and $p < 0.001$ respectively) decreases in phospho-p38 protein levels that began to rebound towards basal untreated levels at 15 and 30 minute treatments with CSG 21680 (Figure 3.10). Because pharmacological inhibition of p38 resulted in decreased PMA/PHA-induced TNF-α protein production in U937 cells, decreased phospho-p38 protein levels induced by A_{2A} receptor activation may be an important mechanism by which CGS 21680 controls TNF-α production. As a control, we measured total p38 levels from U937 cells treated with CSG 21680 for time intervals ranging from 5 to 30 minutes to ensure that the decreased phospho-p38 levels detected at 5 and 10 minute treatments with CGS 21680 did not result from degradation of p38

protein. CGS 21680 treatment for up to 30 minutes did not alter total p38 protein levels in U937 cells (Figure 3.10A).

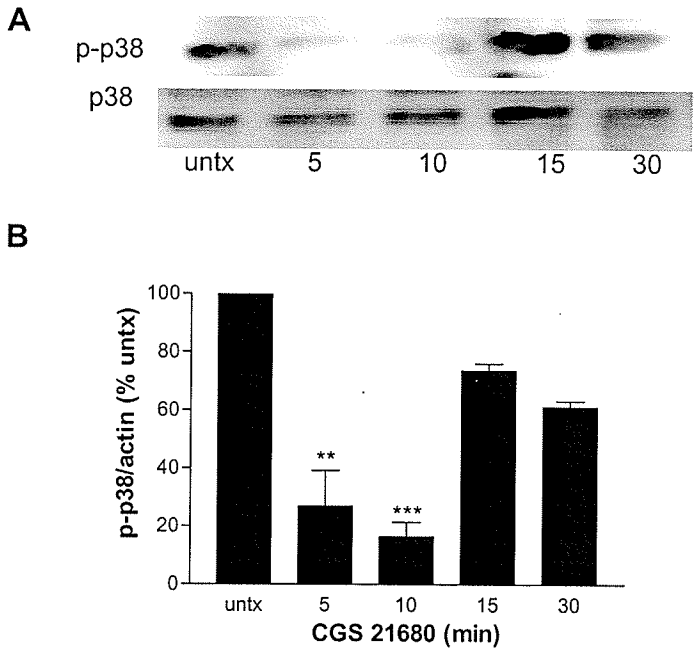

Figure 3.10: Adenosine A_{2A} receptors decrease basal phospho-p38 protein levels. A U937 cells were either untreated (untx) or treated with CGS 21680 (1 μM) for time intervals increasing from 5 to 30 minutes. Phospho-p38 and total p38 levels were determined by western blot with primary antibodies specific for phospho-p38 and total p38. **B** Results from 3 independent experiments are shown graphically. ** $p < 0.01$ vs. untx and *** $p < 0.001$ vs. untx

Activation of p38 by inflammatory stimuli results in activation of transcription factors known to be involved in driving TNF-α gene expression, and consequently p38 may regulate TNF-α production by inducing gene expression. Recently, p38 was identified as an important signaling pathway controlling both general protein translation and TNF-α mRNA stability. Consequently, it was important to determine if p38 regulated TNF-α production in U937 cells pre- or post-transcriptionally. Accordingly, we pre-treated U937 cells with SB 202190 to

block p38, stimulated cells with PMA/PHA for 2 (data not shown) or 4 hours, and measured TNF-α mRNA from cell pellets and TNF-α protein levels from cell culture supernatants. Pre-treatment of U937 cells with SB 202190 did not alter 2 hour (data not shown) or 4 hour (Figure 3.11) PMA/PHA-induced increases in TNF-α mRNA levels suggesting that regulation of TNF-α by p38 was post-transcriptional.

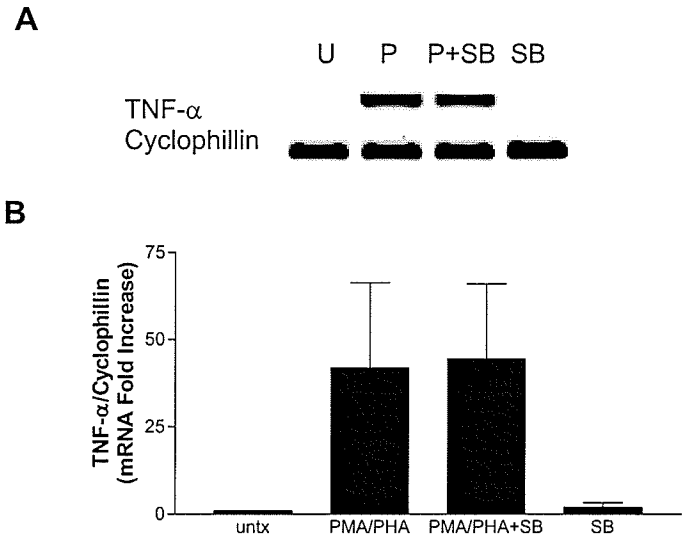

Figure 3.11: p38 does not regulate PMA/PHA-induced TNF-α transcription. A U937 cells were either untreated (untx) or were treated with SB 202190 (1 μM) for 15 minutes prior to stimulation with PMA/PHA for 4 hours. TNF-α mRNA levels were determined by RT-PCR. **B** Results from 3 independent experiments are shown graphically.

In order to verify that p38 regulates PMA/PHA-induced TNF-α production post-transcriptionally, we measured TNF-α protein levels in cell culture supernatants from the same experiments performed to measure TNF-α mRNA levels. Consistent with earlier experiments, pre-treatment of U937 cells with SB 202190 decreased PMA/PHA-induced TNF-α protein production by 58 ± 1% ($p < 0.001$)

following stimulation with PMA/PHA for 2 (data not shown) or 4 hours without altering PMA/PHA-induced increases in TNF-α mRNA (Figure 3.11 and 3.12).

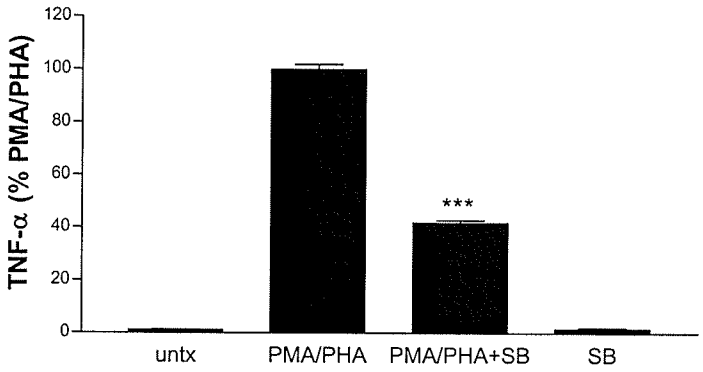

Figure 3.12: p38 regulates PMA/PHA-induced TNF-α protein production. U937 cells were either untreated (untx) or treated with SB 202190 (1 μM) for 15 minutes prior to stimulation with PMA/PHA for 4 hours. TNF-α protein levels in cell culture supernatants were determined by ELISA. *** $p < 0.001$ vs. PMA/PHA

Adenosine A_{2A} receptor activation does not alter activation of p38 dependent proteins involved in translation initiation. Recently, p38 was identified as an important regulator of TNF-α mRNA stability, as well as a more general regulator of protein translation (Kishore et al., 2001; Liu et al., 2002; Mahtani et al., 2001; Manthey et al., 1998; Raught and Gingras, 1999; Rolli-Derkinderen et al., 2003; Rutault et al., 2001; Wang et al., 1999; Wang et al., 1998). Specifically, p38 activation results in phosphorylation of the eukaryotic initiation factor eIF-4E and its inhibitory binding protein 4EBP1 (Liu et al., 2002; Potter et al., 2001; Raught and Gingras, 1999; Rolli-Derkinderen et al., 2003; Wang et al., 1998). Phosphorylation of these proteins results in dissociation, binding of eIF-4E to the 5' cap of mRNA, and recruitment of a translation initiation protein complex.

Because p38 regulates PMA/PHA-induced TNF-α protein production post-transcriptionally, and because CGS 21680 decreases phosphorylation of p38, we hypothesized that activation of A_{2A} receptors with CGS 21680 would decrease phosphorylation of eIF-4E and 4EBP1. To test this hypothesis, U937 cells were treated with CGS 21680 for time intervals ranging from 5 to 60 minutes and phospho-eIF4E and phospho-4EBP1 protein levels were determined by western blot. CGS 21680 treatment of U937 cells had no effect on phospho-eIF4E or phospho-4EBP1 protein levels at any of the time points tested, suggesting that this p38 activated signaling pathway which regulates protein translation is not a target of adenosine A_{2A} receptor activation (Figure 3.13).

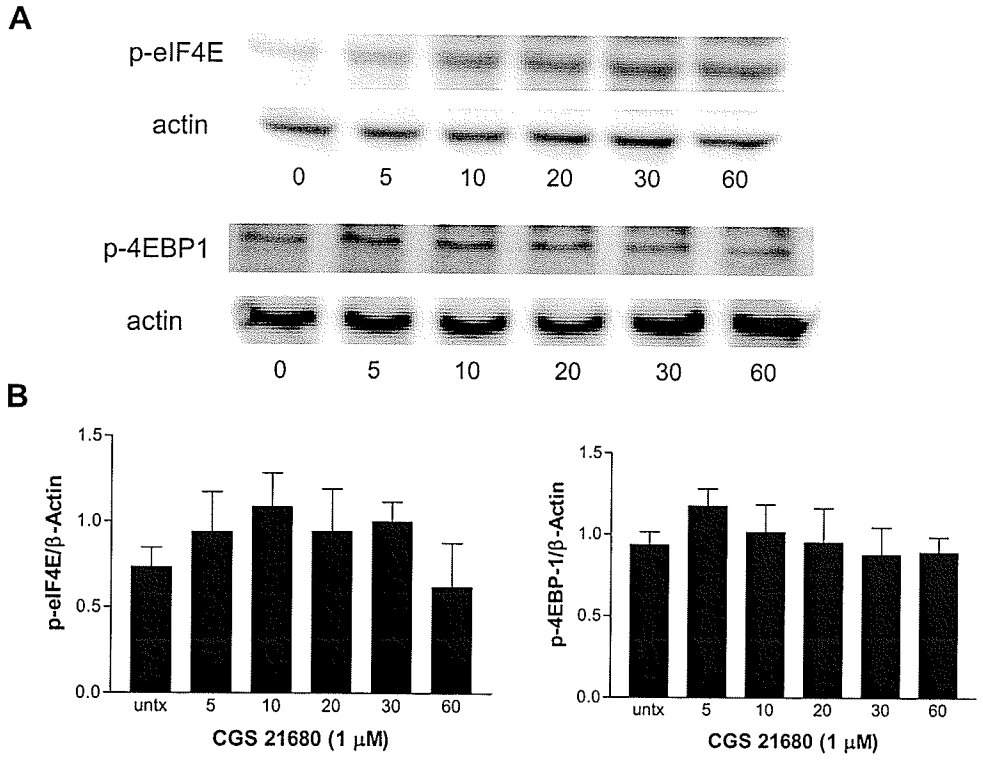

Figure 3.13: Adenosine A_{2A} receptors do not affect phospho-eIF4E or phospho-4EBP1 protein levels. **A** U937 cells were either untreated (untx) or treated with CGS 21680 (1 μM) for time intervals ranging from 5 to 60 minutes and p-eIF4E and p-4EBP1 protein levels were determined by western blot. **B** Results from 3 independent experiments are shown graphically.

Activation of adenosine A_{2A} receptors does not decrease basal phospho-JNK levels. In addition to ERK and p38, JNK is important to PMA/PHA-induced TNF-α production because the JNK inhibitor SP 600125 blocked TNF-α production by 71 ± 2% (Figure 3.3). However, like p38, protein levels of phospho-JNK were not increased by PMA/PHA suggesting that basal levels of active JNK were sufficient for regulating TNF-α (Figure 3.8). As a result, we hypothesized that activation of adenosine A_{2A} receptors would decrease basal levels of phospho-JNK as a mechanism for regulating TNF-α production. To test this hypothesis, we treated U937 cells with CGS 21680 for time intervals ranging from 5 to 60 minutes and measured phospho-JNK protein levels by western blot. Activation of adenosine A_{2A} receptors for up to 60 minutes had no effect on basal levels of phospho-JNK, suggesting that activity of the JNK signaling pathway was not affected by CGS 21680 (Figure 3.14).

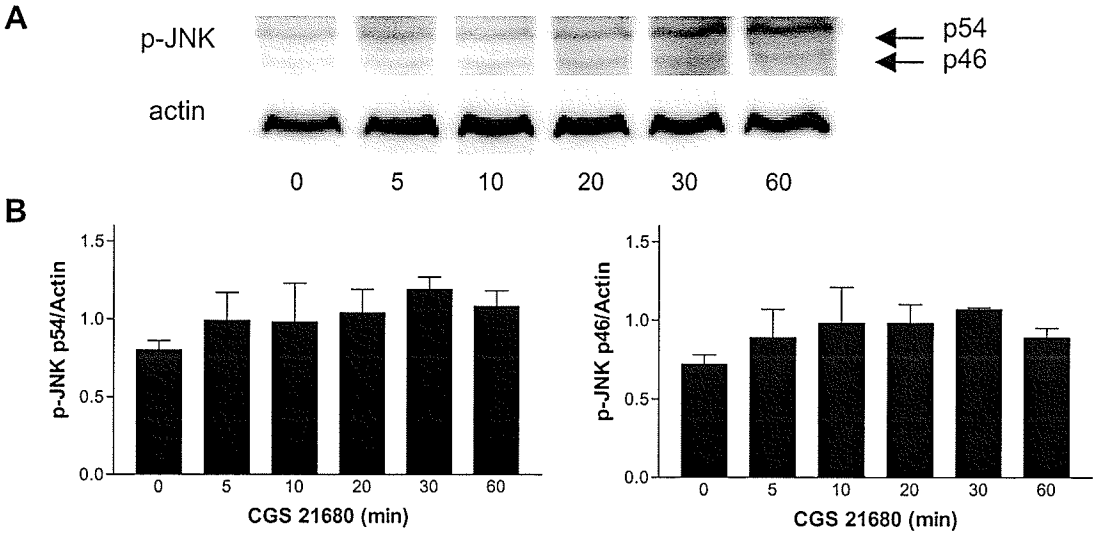

Figure 3.14: Adenosine A_{2A} receptors do not affect phospho-JNK protein levels. **A** U937 cells were either untreated (0) or treated with CGS 21680 (1 μM) for time intervals ranging from 5 to 60 minutes and p-JNK protein levels were determined by western blot. **B** Results from 3 independent experiments are shown graphically.

Discussion

Several groups have studied the regulation of cytokine production by adenosine and adenosine receptor agonists, however the cellular signaling mechanisms involved in mediating these effects are not well understood. Accordingly, we sought to identify signal transduction mechanisms involved in mediating post-transcriptional regulation of TNF-α production by adenosine A_{2A} receptors.

Adenosine A_2 receptors are coupled to Gs proteins, which increase intracellular cAMP production through adenylyl cyclase when the receptors are activated and subsequently activate cAMP dependent signaling pathways like PKA. We tested the hypothesis that a cAMP/PKA-dependent signaling pathway mediated the anti-TNF-α effects of adenosine A_{2A} receptors. We found that the anti-TNF-α effect of CGS 21680 was independent of cAMP production and PKA activity because neither a cAMP antagonist nor a PKA inhibitor reversed the inhibition of TNF-α production mediated by A_{2A} receptor activation. Our findings are similar to those by Sajjadi et al., who showed that the anti-TNF-α effects of the adenosine receptor agonist I-ABA were independent of cAMP and adenylyl cyclase in LPS-stimulated U937 cells (Sajjadi et al., 1996), however I-ABA is an A_1/A_3 receptor agonist and both of these receptors are coupled to Gi proteins, which inhibit adenylyl cyclase and decrease cellular levels of cAMP. However, our findings differ from other studies which showed that the anti-TNF-α effect of adenosine in neonatal rat myocytes was blocked by the adenylyl cylcase inhibitor MDL 12330A (Wagner et al., 1998). Bshesh et al. also showed that the anti-

TNF-α effect of adenosine involved the cAMP/PKA pathway in the monocytic cell line THP-1 (Bshesh et al., 2002). Both of these studies used adenosine, which can activate all 4 receptor subtypes and all 4 receptor subtypes can block TNF-α production, however the involvement of the cAMP/PKA pathway in the anti-TNF-α effects of adenosine suggest primarily A_2 receptor mediated events. The studies implicating cAMP/PKA in mediating the anti-TNF-α effects of adenosine were performed using myocytes and monocytes. Although these studies suggest A_2 receptor mediated events, contrasting results from our studies combined with those from other groups suggest that like regulation of TNF-α mRNA and protein, involvement of cAMP/PKA signaling pathways in mediating the anti-TNF-α effect of adenosine receptors may be cell-type and stimulus specific and may also result from different A_{2A} receptors coupling to G proteins or to effector molecules.

Production of TNF-α in macrophages depends on activation of MAPK signaling pathways, specifically p38, ERK, JNK, and BMK/ERK5. Stimulating macrophages with LPS causes phosphorylation and activation of ERK, p38 and JNK (Dumitru et al., 2000; Means et al., 2000; Rao, 2001; Swantek et al., 1997; Zhu et al., 2000) whereas PMA stimulation results in phosphorylation of ERK (Rao, 2001). Activated MAPK proteins phosphorylate and activate various transcription factors including NF-κB, Elk1, Egr1 and ATF-2/Jun (Fuchs et al., 2000; Paludan et al., 2001; Rao, 2001; Shi et al., 2002; Tsai et al., 2000; Udalova and Kwiatkowski, 2001) that drive transcription of inflammatory genes including TNF-α (Paludan et al., 2001; Tsai et al., 2000; Tsai et al., 1996; Udalova and

Kwiatkowski, 2001). In addition, MAPK pathways have also been shown to induce TNF-α expression by acting on RNA polymerase II (Zhu et al., 2000). Recently, MAPK signaling molecules have been implicated in controlling protein translation in addition to their well-described transcriptional effects (Brook et al., 2000; Knauf et al., 2001; Lee et al., 2000; Mahtani et al., 2001; Rutault et al., 2001; Swantek et al., 1997; Wang et al., 1998). In particular, p38 has been implicated in regulating TNF-α RNA stability (Brook et al., 2000; Liu et al., 2000; Mahtani et al., 2001; Rutault et al., 2001) and activation of protein translation (Knauf et al., 2001; Liu et al., 2002; Wang et al., 1998). Accordingly, we designed experiments to identify the MAPK signaling pathways involved in regulating TNF-α production in PMA/PHA-stimulated macrophages and which of these pathways were targets of adenosine A_{2A} receptor activation. We found that the activity of the MAPK signaling molecules p38, ERK, JNK and PI3K were important for PMA/PHA-induced increases of TNF-α in U937 cells, with p38, JNK and ERK inhibitors having the most profound anti-TNF-α effect of the MAPK inhibitors tested. In addition, the phospholipase C inhibitor dose-dependently inhibited TNF-α production, implicating this signaling pathway in regulating TNF-α production. This finding is consistent with studies from our group showing the involvement of the phospholipase C pathway in regulating TNF-α induced by the HIV protein Tat in human macrophages (unpublished data). Phospholipase C may regulate TNF-α production by increasing intracellular calcium levels resulting from inositol trisphosphate production.

Consistent with other studies, only ERK phosphorylation was significantly increased by PMA/PHA, however these increased levels of phospho-ERK were not blocked by activation of A_{2A} receptors with CGS 21680. This finding is consistent with other studies in monocytes and macropahges, which showed that the anti-TNF-α effects of adenosine and adenosine receptors were not mediated by blocking the ERK signaling pathway (Bshesh et al., 2002; Hasko et al., 2000; Sajjadi et al., 1996). However, activation of A_{2A} receptors in non-immune cells such as CHO cells, HEK293 cells, and endothelial cells has been shown to activate ERK and ERK-dependent signaling pathways. The functional effects of ERK activation by A_{2A} receptor activation have only been studied in endothelial cells and are involved in stimulating endothelial cell growth, an effect that may participate in long-term protection following ischemia or hypoxia by promoting angiogenesis (Klinger et al., 2002). These studies emphasize the cell-specific nature of both functional effects of adenosine A_{2A} receptor activation and the probable differences in receptor coupling to downstream signaling effectors among different cell types.

In addition to ERK, p38 was particularly important for regulation of PMA/PHA-induced TNF-α production although PMA/PHA did not increase phospho-p38 levels over basal levels in U937 cells. p38 has been implicated in post-transcriptional regulation of TNF-α and as such may be a target of adenosine A_{2A} receptor activation. We hypothesized that basal levels of p38 activity in untreated cells were required for production of TNF-α induced by PMA/PHA and

as such, were a target of intervention for adenosine A_{2A} receptors. Treatment of U937 cells with CGS 21680 for 10 to 15 minutes decreased basal phospho-p38 protein levels, which began to rebound towards normal levels at 30 minutes, whereas CGS 21680 had no effect on basal protein levels of phospho-JNK. In addition, we showed that the p38 inhibitor SB 202190 decreased PMA/PHA-induced increases in TNF-α protein production without decreasing steady-state TNF-α mRNA levels. These data suggest that, as indicated by others, p38 regulates mRNA stability and/or protein translation (Kishore et al., 2001; Kleijn et al., 1998; Knauf et al., 2001; Lee et al., 2000; Liu et al., 2002; Mahtani et al., 2001; Manthey et al., 1998; Means et al., 2000; Raught and Gingras, 1999; Rolli-Derkinderen et al., 2003; Rutault et al., 2001; Wang et al., 1999; Wang et al., 1998) in our model and like CGS 21680 does not regulate TNF-α production by activating transcription factors and initiating TNF-α gene expression.

Although the effect of CGS 21680 on phospho-p38 levels was transient and began to recover to basal levels after 30 minutes, the downstream effects of this decrease in p38 activity may be longer lasting and may contribute to the anti-TNF-α effect of CGS 21680 which is maintained for 4 to 8 hours following PMA/PHA stimulation. Because p38 coordinates with several important signaling pathways for full induction of TNF-α production in macrophages (Zhu et al., 2000), even short, transient de-phosphorylation of p38 may have longer lasting downstream effects that could impact on TNF-α protein production. Specifically, activation of protein targets phosphorylated by p38 will be affected as a

secondary consequence of A_{2A} receptor activation and will require time to regain function to override the effect of CGS 21680. Furthermore, the MAPK signaling pathways that coordinate to control TNF-α production (p38, ERK, JNK, BMK) are not distinct and have several proteins in common that either activate or are activated by MAPK signaling molecules. Thus, p38 de-phosphorylation may also affect signaling molecules that participate in ERK and JNK signaling, leading to downstream effects that last longer than the transient decrease in phospho-p38 induced by CGS 21680 and contribute to the anti-TNF-α effect of A_{2A} receptors.

In our model, p38 activity was important for regulating TNF-α production post-transcriptionally because the p38 inhibitor SB 202190 blocked PMA/PHA-induced TNF-α protein production without affecting TNF-α mRNA levels. Recently, p38 was identified as a signaling molecule involved in regulating protein translation by activating the eukaryotic initiation factor eIF-4E pathway. p38 phosphorylated both eIF-4E (Knauf et al., 2001; Rao, 2001; Wang et al., 1998) and its inhibitor binding protein 4EBP1 (Liu et al., 2002). Phosphorylation of 4EBP1 releases this inhibitory protein from eIF-4E, allowing eIF-4E to interact with the 5' end of mRNA and recruit translational machinery to the site (Knauf et al., 2001; Wang et al., 1998). Phosphorylation of eIF-4E by p38 may increase its affinity for mRNA (Wang et al., 1998). We hypothesized that by decreasing p38 phosphorylation, adenosine A_{2A} receptors may decrease the activity of a signaling pathway involved in protein translation by blocking phosphorylation of eIF-4E and 4EBP1. Our data shows that activation of A_{2A} receptors with CGS 21680 does not

decrease the basal levels of phospho-eIF4E and phospho-4EBP1, suggesting that A_{2A} receptors do not control TNF-α production by blocking translation through recruitment of this initiation complex to the 5' cap of mRNA.

In addition to controlling translation, p38 has also been implicated in controlling TNF-α mRNA stability (Brook et al., 2000; Mahtani et al., 2001; Rutault et al., 2001). Our data showed no attenuation of PMA/PHA-induced increases in TNF-α mRNA levels with either SB 2021290 or CGS 21680 suggesting that p38 is involved in translation of TNF-α rather than stabilizing TNF-α mRNA.

Finally, we investigated the mechanism by which adenosine A_{2A} receptor activation may block activity of MAPK signaling pathways, particularly p38, and/or other signaling pathways involved in TNF-α production as a mechanism for controlling TNF-α production. MAPK signaling pathways and other signaling pathways like PI3K and PLC are controlled by phosphorylation. In general, signaling proteins are in their active state when phosphorylated on serine, threonine, and/or tyrosine residues. As a result, the relay of intracellular signals is balanced by the activities of kinases and phosphatases. We hypothesized that activation of adenosine A_{2A} receptors would block TNF-α production by activating a protein phosphatase. Using pharmacological approaches, our data showed that inhibitors of serine/threonine and tyrosine phosphatases can partially reverse the anti-TNF-α effects of CGS 21680, with complete reversal seen with the highest concentration of okadaic acid tested, suggesting the involvement of the

serine/threonine phosphatase PP1. We went on to directly measure serine/threonine phosphatase activity in extracts of U937 cells treated with the adenosine A_{2A} receptor agonist. One study investigating the involvement of protein phosphatase in the actions of A_{2A} receptors on neutrophils identified PP1 as an enzyme whose activity is increased by the non-selective A_1/A_2 receptor agonist NECA only in plasma membrane fractions of human neutrophils with a small decrease in activity detected in cytosolic fractions (Revan et al., 1996). This study also found that the effect of NECA on free radical production in neutrophils was reversed by serine/threonine phosphatase inhibition, suggesting that A_2 receptors control neutrophil function by activating plasma membrane serine/threonine phosphatase. Like this study, our data showed that A_{2A} receptor activation increased serine/threonine phosphatase activity in plasma membrane fractions, however we detected this increase after 30 to 60 minutes, whereas the neutrophil study detected increased activity after 5 minutes. In contrast to the study on neutrophils, our data showed an almost immediate increase in enzyme activity in cytosolic fractions of U937 cells after 5 to 10 minutes of CGS 21680, whereas a small decrease in cytosolic activity was measured after 5 minutes in neutrophils.

In summary, production of TNF-α in U937 cells induced by PMA/PHA required activity of MAPK cell signaling pathways as observed by others in monocytes and macrophages. Activity of these cell signaling pathways is controlled by phosphorylation which is mediated by cascades of kinase and phosphatase

activity within a cell. Because control of TNF-α is regulated by activation of the specific pathways identified, kinase activity dominates to phosphorylate the proteins involved in signal transmission, mRNA stability, and protein translation. In order to control TNF-α production, adenosine A_{2A} receptor activation results in increased serine/threonine phosphatase activity, which may assist in controlling activation of or turning off the required signaling pathway(s). Our data suggest that adenosine A_{2A} receptor activation decreases phosphorylation and activity of a p38 dependent signaling pathway involved in controlling TNF-α mRNA stability, causing a decrease in mRNA half-life and stability. The observed decrease in phospho-p38 may result from increased serine/threonine phosphatase activity initiated by adenosine A_{2A} receptors. A schematic diagram summarizing the data presented in this chapter is shown in Figure 3.15.

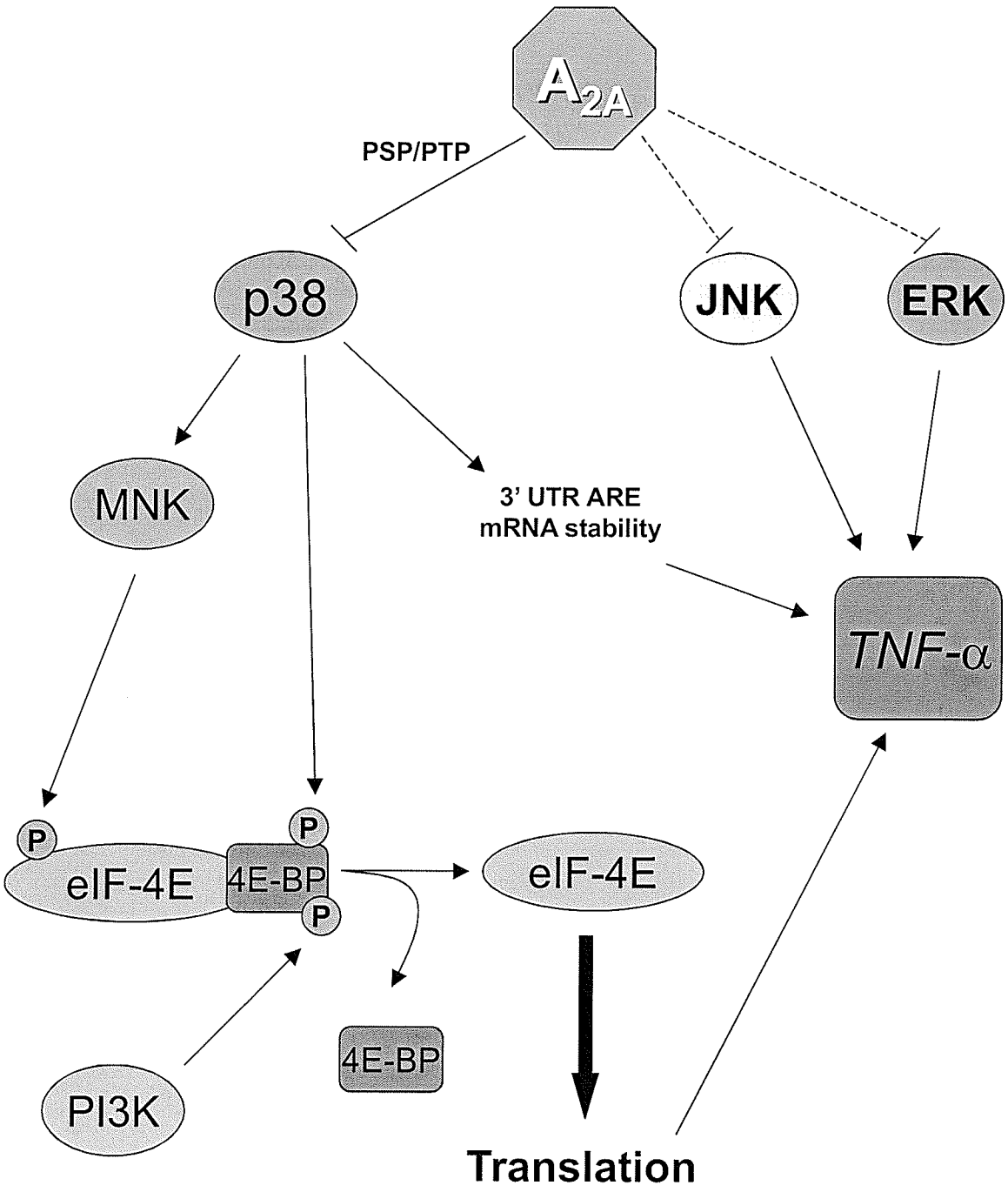

Figure 3.15: Adenosine A_{2A} Receptor-mediated Signaling. Schematic diagram showing cell signaling pathways involved in TNF-α production and A_{2A} receptor-mediated anti-TNF-α effects. Solid arrows show activation, solid lines with blunt end show inhibition, dashed lines show experiments conducted with no effect observed.

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Chapter 4

Preliminary Studies of the Effects of Adenosine Receptor Activation on Inflammation and the Immune System: Focus on Multiple Sclerosis, Cell Adhesion, and DNA Microarray Studies

Effects of Adenosine Receptors in Multiple Sclerosis and on Monocyte Adhesion.

Introduction

Multiple sclerosis (MS) is an inflammatory, degenerative disorder of the central nervous system (CNS). The pathological hallmarks of MS include lesions within brain white matter that are associated with demyelination, local inflammation, and loss of oligodendrocytes that normally control myelination and are the primary cells lost within the CNS during MS (Ludwin, 2000). Although the causes of this disorder remain elusive, there is strong evidence suggesting three potential triggers and/or exacerbation factors: autoimmunity, excitotoxicity, and viral infection.

The presence of activated T cells in MS that recognize myelin basic protein has contributed to the theory that MS is an autoimmune disease (Ludwin, 2000). An association of viruses like human herpes virus type 6 with MS lesions has contributed to the theory that MS has a viral component (Ablashi et al., 1998; Akhyani et al., 2000; Alvarez-Lafuente et al., 2002; Berti et al., 2000; Challoner et al., 1995; Friedman et al., 1999; Ongradi et al., 1999; Sanders et al., 1996; Soldan et al., 1997; Soldan and Jacobson, 2001; Soldan et al., 2000; Tejada-Simon et al., 2002). Animal studies of experimental autoimmune encephalomyelitis (EAE) showing increased glutamate levels and protection against the deleterious effects of EAE with AMPA/kainate receptor blockers

support the theory that excitotoxicity contributes to the development of MS (Matute et al., 2001; Ohgoh et al., 2002; Pitt et al., 2000; Werner et al., 2000).

A common theme among the three theories identified above is the involvement of activated T lymphocytes that infiltrate the CNS and contribute to the development of MS lesions. T lymphocytes from the peripheral circulation become activated by unknown mechanisms and cross the blood brain barrier (BBB) into the brain parenchyma. Migration across the BBB is mediated by increases in matrix metalloproteinases and expression of adhesion molecules like ICAM-1, VCAM-1, and E-selectin on endothelial cells. Within the brain, antigen-presenting cells like astrocytes, microglia, and macrophages through MHCII-presented antigen activate T lymphocytes. Stimulation of T cell receptors by antigen-presenting cells results in production of cytokines that mediate primarily TH1 immune responses, like TNF-α and IL-12 (Keegan and Noseworthy, 2002). Kouwenhoven and colleagues showed recently that monocytes may also play a prominent role in MS because blood-derived monocytes isolated from MS patients secreted increased basal and stimulated levels of IL-12, a TH1 cytokine, and IL-6, a cytokine with an unknown role in MS that normally controls B and T lymphocyte differentiation and production of soluble cytokine-binding receptors (Kouwenhoven et al., 2001). This group also identified increased expression of co-stimulatory molecules like CD86 on monocytes isolated from MS patients, suggesting that these cells may have a greater capacity to activate T lymphocytes than those from healthy individuals. In addition, activated

monocytes are abundant in MS lesions and as such may play an important role in demyelination and may facilitate T lymphocyte migration into the CNS by secreting cytokines.

Several studies have shown that TNF-α levels (Mayne et al., 1999) are increased during MS however the role that this cytokine plays in the development of the disease is unclear. Experimental evidence has suggested that TNF-α contributes to demyelination and loss of oligodendrocytes. Neutralization of TNF-α has been beneficial in EAE, however clinical trials with TNF-α antibodies resulted, surprisingly, in exacerbation of the disease and more severe neurological deficit, suggesting a potential beneficial role of TNF-α (1999). TNF-α helps mediate differentiation and myelination by oligodendrocytes and oligodendrocyte precursor cells (Arnett et al., 2001) and it appears that TNF-α may play several roles in MS, both beneficial and destructive. Endogenous mechanisms that regulate its production are therefore important in understanding the contribution of cytokines to the development and progression of MS.

Work by others and us has identified the adenosine system as an important endogenous regulator of immune responses and cytokine production. As such, adenosine and adenosine receptors may play a role in the development of MS or may provide novel therapeutic targets for its treatment. Decreased density and function of adenosine A_1 receptors has been demonstrated in both blood and brain from MS patients (Johnston et al., 2001; Mayne et al., 1999). Decreased

plasma adenosine levels have been observed in MS patients in addition to findings of impaired anti-TNF-α effects of the adenosine A_1 receptor agonist R-PIA in stimulated peripheral blood mononuclear cells from MS patients (Mayne et al., 1999). Adenosine A_1 receptor expression was also found to be decreased on monocytes and macrophages in both brain and blood of MS patients (Johnston et al., 2001).

As a result of findings implicating TNF-α and adenosine receptors in MS, we studied the anti-TNF-α effects of the adenosine receptor system in macrophages isolated from MS patients and healthy controls. Because monocyte migration into the CNS is an important factor contributing to MS, we also investigated the ability of adenosine receptors to modulate monocyte adhesion.

Methods

TNF-α assays and drug treatments were performed in the same manner as with U937 cells discussed in the Methods and Results sections of Chapter 3. Detailed protocols for all experimental techniques in this Chapter can be found in the Appendix. Briefly, monocytes were isolated from blood collected from MS patients or healthy volunteers and were allowed to differentiate for 7 days on plastic tissue culture plates. Drugs were prepared in RPMI 1640 media and added to primary macrophage cell cultures prior to stimulation. Cell cultures were stimulated with LPS (100 ng/ml) for 4 hours, supernatants were collected, and TNF-α protein levels were determined by ELISA.

To study adenosine receptor modulation of monocyte adhesion, THP-1 cells were treated with adenosine receptor agonists for 30 minutes prior to determination of fibronectin-mediated binding. Cell adhesion was determined as described in the Appendix.

Preliminary Results and Discussion

Adenosine receptor modulation of TNF-α in macrophages from MS patients and healthy controls. Two studies suggest that decreased expression of adenosine A_1 receptors may occur in blood and brain in MS (Johnston et al., 2001; Mayne et al., 1999). Macrophages and monocytes are the primary producers of TNF-α, decreased adenosine A_1 receptor expression in MS is primarily on cells of monocytic lineage (Johnston et al., 2001), adenosine A_1 receptors control TNF-α production, and TNF-α may contribute to both remyelination and lesion development in MS (Arnett et al., 2001). Consequently, we studied the differences in anti-TNF-α effects of adenosine receptor activation in macrophages isolated from MS patients and healthy controls (Figure 4.1). We investigated the effects of adenosine A_{2A} receptor activation with CGS 21680 and A_1 receptor activation with R-PIA by pre-treating macrophages with these agents for 30 minutes prior to LPS challenge for 4 hours. Notably, there was no significant difference in levels of TNF-α produced by LPS in macrophages from MS patients and healthy controls. Activation of adenosine A_{2A} receptors with CGS 21680 significantly inhibited LPS-induced TNF-α production in macrophages isolated from MS patients ($p < 0.05$) and healthy controls ($p < 0.01$), but no statistically significant differences in the degree of inhibition were observed between MS patients and healthy controls. In contrast, activation of A_1 receptors with R-PIA significantly decreased LPS-induced induced increases in TNF-α production in macrophages isolated from healthy controls, but not from MS patients. Whereas the A_{2A} antagonist ZM 241385 reversed the anti-TNF-α

effects of CGS 21680, the A_1 antagonist DPCPX did not reverse the anti-TNF-α effects of R-PIA. This suggests that other effects, including actions on other adenosine receptors, may mediate the anti-TNF-α effects of R-PIA. Further studies with other A_1 agonists and antagonists will be required to help elucidate the role of A_1 receptor activation in controlling TNF-α production in macrophages from both MS patients and healthy controls.

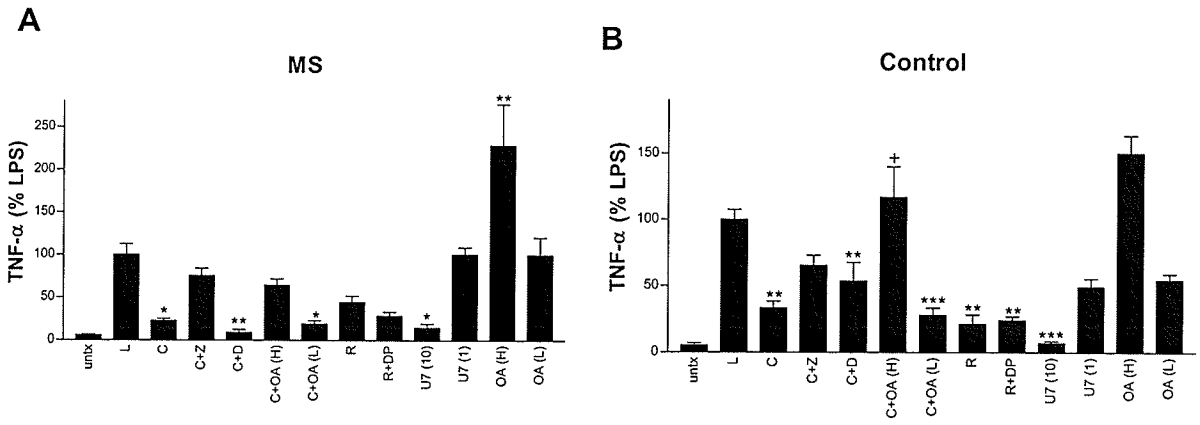

Figure 4.1: Adenosine receptor modulation of TNF-α in MS. A Macrophages from MS patients were either untreated (untx) or treated with lipopolysaccharide (L) alone or in combination with CGS 21680 (C; 1 μM), ZM 241385 (Z; 100 nM), dephostatin (D; 1 μM), okadaic acid (OA; H=100 nM, L=1 nM), R-PIA (R; 1 μM), DPCPX (DP;), or U73122 (U7; μM). After 4 hours, supernatants were collected and TNF-α levels were determined by ELISA. **B** Identical experiments performed in macrophages from healthy controls. For both graphs, * $p < 0.05$ vs. LPS, ** $p < 0.01$ vs. LPS, *** $p < 0.001$ vs. LPS, and + $p < 0.001$ vs. L+C

To determine whether the signaling mechanisms mediating A_{2A} receptor actions in macrophages are conserved during a chronic inflammatory disease, we studied the involvement of protein phosphatase in mediating the anti-TNF-α effects of A_{2A} receptors in macrophages from MS patients and healthy controls. Treatment of MS macrophages with 100 nM okadaic acid significantly ($p < 0.01$)

increased protein levels of TNF-α over those induced by LPS alone but had no significant effect on LPS-induced TNF-α levels in healthy macrophages. The anti-TNF-α effects of CGS 21680 were significantly ($p < 0.001$) reversed by okadaic acid at 100 nM but not 1 nM in healthy macrophages, suggesting the involvement of PP1 in macrophages as in U937 cells. However, the anti-TNF-α effects of CGS 21680 were not significantly reversed by okadaic acid in MS macrophages. These data suggest that PP1 and PP2A are not involved in A_{2A} receptor mediated inhibition of TNF-α in MS macrophages whereas the anti-TNF-α effects of A_{2A} receptors in healthy macrophages involve serine/threonine phosphatase activity. Although A_{2A} receptors are expressed and exhibit anti-TNF-α actions in macrophages from MS patients and healthy controls, the signaling pathways coupled to A_{2A} receptors may be altered during chronic inflammatory disease.

During MS, monocytes are activated and recruited into the CNS where they differentiate and contribute to disease pathology by producing cytokines and chemokines. As a result of a chronic inflammatory disease, the signaling events mediating cytokine and chemokine production may be more active or hyperresponsive to an inflammatory stimulus compared to healthy macrophages. In addition, hyperresponsive phosphorylation-dependent signaling pathways may be associated with hyperactivated phosphatase activity in an effort to control the response of a macrophage to an inflammatory stimulus. Aberrant signaling pathways in MS may result in high levels of serine/threonine phosphatase activity

that when blocked by okadaic acid result in TNF-α protein levels significantly higher than those induced by LPS alone. To test this hypothesis, serine/threonine phosphatase activity can be measured, as in U937 cells, in monocytes and macrophages isolated from MS patients and healthy controls. Higher phosphatase activity in MS macrophages may help explain the effect of okadaic acid on LPS-induced TNF-α production. This theory, however, does not explain why the anti-TNF-α effects of CGS 21680 on macrophages were not significantly reversed by okadaic acid in MS macrophages. Although pharmacological evidence with okadaic acid implicates PP1 in both MS and healthy macrophages, the specific subunits mediating the effects of PP1 have not been identified in human macrophages. Differences in subunit expression in healthy versus MS macrophages may explain augmentation of LPS-induced TNF-α production without reversing the anti-TNF-α effects of CGS 21680 as different subunit expression can confer different specificities for target proteins. In addition to studying differences in phosphatase activity in macrophages from MS patients and healthy controls, more clearly defined examination of the role of adenosine receptors in mediating production of TNF-α and other cytokines involved in MS like IL-12 will be important in determining the interaction between MS and the adenosine system. Further experiments studying dose-response curves, antagonist actions and using newer, highly selective agonists will help identify specific actions of adenosine receptors on cytokine production in MS. Finally, very little is known about the specific coupling of adenosine receptors to G proteins and G protein subunits in immune cells. Differences in coupling of

receptors to downstream effector pathways may influence the anti-TNF-α effects of adenosine receptors in macrophages from MS patients and healthy controls.

Because the macrophages we studied in these experiments were isolated as blood-derived monocytes and were differentiated for 7 days, it will be important to determine differences in adenosine receptor expression during the differentiation process. Also, adenosine has been proposed as a potential mediator of MS because it can stimulate differentiation of oligodendrocyte precursor cells into mature, myelin producing oligodendrocytes (Stevens et al., 2002). Studies examining the role of adenosine receptors in monocyte and macrophage differentiation may provide invaluable information to understand how adenosine controls immune responses because of the well documented effects of adenosine on macrophage function normally, and now possibly during MS.

Adenosine receptor-mediated modulation of monocyte cell adhesion. An early step in the activation of monocytes leading to their migration into the CNS and differentiation into macrophages, which in turn produce and release pro-inflammatory cytokines, is adhesion of monocytes to endothelial cells that form part of the blood brain barrier (BBB). In addition to controlling cytokine production, we hypothesized that adenosine may modulate monocyte and macrophage function by decreasing adhesion of monocytes. To test this hypothesis, we established a model to induce binding of THP-1 cells to fibronectin coated cell culture plates. Binding of monocytes to extracellular

proteins like fibronectin initiates monocyte/macrophage activation and differentiation. Recent studies have shown that adenosine decreased $\alpha 4\beta 7$ integrin-mediated binding of T lymphocytes to colon adenocarcinoma cells (MacKenzie et al., 2002) but $\alpha 4$ integrins are also involved in binding to fibronectin. Activation of monocytes by factors like cytokines or chemokines can increase monocyte adhesion by activating intracellular signaling pathways including second messengers like PKC and intracellular Ca^{2+}. We conducted experiments to determine if THP-1 cells provided an in vitro model of monocyte adhesion by studying monocyte binding to fibronectin using PMA to activate PKC, ionomycin to increase intracellular Ca^{2+} levels. Cell cultures were treated with PMA, ionomycin, or LPS for 15 minutes prior to determining cell adhesion to fibronectin. Neither PMA nor ionomycin increased fibronectin specific binding of THP-1 cells, however LPS stimulation increased binding 2.2 ± 1.4 fold over binding of untreated cells (Figure 4.2).

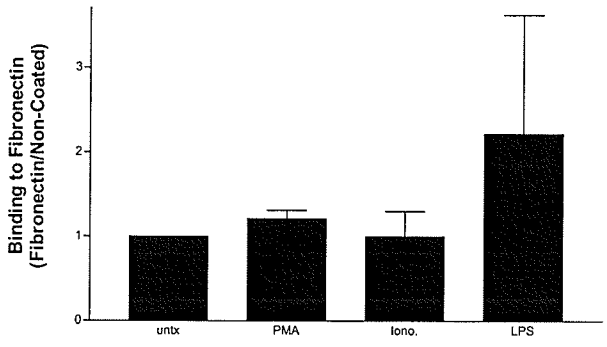

Figure 4.2: LPS increases monocyte binding to fibronectin. THP-1 cells were either untreated (untx) or were treated with PMA (10 ng/ml), ionomycin (100 nM), or LPS (100 ng/ml) for 15 minutes before cell adhesion to fibronectin was measured. Data are represented as fold change from untx of ratios of fibronectin binding over non-specific binding to plastic culture plates.

To examine whether adenosine receptor activation decreased fibronectin-mediated binding in monocytes, we initially studied the effect of adenosine receptor agonists on basal levels of binding in unstimulated THP-1 cells. Cell cultures were treated with adenosine receptor agonists for 30 minutes prior to determination of binding in unstimulated cultures. Activation of A_{2A} receptors with CGS 21680, at a receptor-selective concentration (Klotz, 2000), significantly decreased binding to fibronectin by 31 ± 4% ($p < 0.001$), whereas activation of A_1 receptors with CCPA and A_3 receptors with AB-MECA had no significant effect on binding of monocytes to fibronectin although there was a trend towards inhibition by A_1 and A_3 receptor activation (Figure 4.3). These results implicate a role for adenosine A_{2A} receptors in regulating monocyte adhesion. CGS 21680 binds to human A_{2A} receptors with Ki = 27 nM and to A_1 receptors with Ki = 67 nM (Klotz, 2000). Although CGS 21680 at a concentration of 40 nM is selective for A_{2A} receptors based on these data, binding studies with lower concentrations of CGS 21680 and with the A_{2A} antagonist ZM 241385 will fully determine the role of A_{2A} receptor activation in controlling monocyte adhesion.

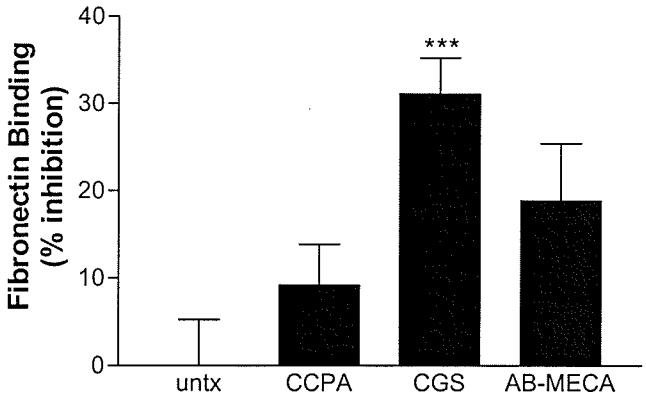

Figure 4.3: Activation of adenosine A_{2A} receptors decreases monocyte binding to fibronectin. THP-1 cells were either untreated (untx) or were treated with CCPA (10 nM), CGS 21680 (40 nM) or AB-MECA (100 nM) for 30 minutes prior to determination of fibronectin-mediated binding. *** $P < 0.001$ vs. untx

Further studies examining the time courses of adenosine receptor agonist effects on fibronectin-mediated binding and the effects on integrin expression will help to elucidate the role of adenosine receptors in controlling monocyte adhesion. The potential importance of these studies is highlighted by early studies in EAE mice that showed that blocking α4 integrin could reduce paralysis (Yednock et al., 1992). Data from clinical trials indicate that an antibody directed against α4 integrins can reduce clinical attacks in MS patients (Miller et al., 2003). Because few endogenous inhibitors of integrin function have been identified, discovery of adenosine-mediated decreases in fibronectin binding and the mechanisms by which these effects occur will further our understanding of how immune cells migrate into tissues and may provide important and novel therapeutic targets for disorders like multiple sclerosis.

Effect of Adenosine A_{2A} Receptor Activation on Immune Gene Expression

Introduction

To date, experiments conducted by others and us studying the immune regulatory effects of adenosine have focused on regulation of individual cells and specific functions of those cells like production of the pro-inflammatory cytokines TNF-α, IL-12 and IL-6. Accordingly, it was proposed that adenosine may act as a general regulator of immune responses (Sitkovsky, 2003). However, to date no studies have been published studying the large-scale effects of adenosine on a broad spectrum of genes involved in immune responses simultaneously using DNA microarray technology. Using a novel immune gene microarray, we performed experiments investigating the role of adenosine A_{2A} receptors in regulating expression of genes encoding proteins involved in the immune system including cytokines, chemokines, adhesion molecules, receptors, and cell signaling molecules. Our findings reinforce the potential importance of adenosine receptor systems in mediating immune cell adhesion, the involvement of p38 signaling pathways in controlling immune cell function, and findings by others indicating that adenosine receptor activation mediates neutrophil function.

Although work by others and us have shown that A_{2A} receptor activation regulates TNF-α in a post-transcriptional manner, microarray technology may provide important clues towards our understanding of general immune responses regulated by adenosine receptor systems. Future experiments designed to investigate the effects of A_{2A} receptors on large-scale immune protein expression

will complete the picture these preliminary experiments have begun to paint. Although to date we have only investigated the effects of A_{2A} receptors on immune gene expression, A_1 and A_3 receptor agonists will also provide important information for general immune regulation by adenosine receptor systems at different stages of inflammation and tissue damage.

Methods

We conducted preliminary experiments using monocyte-derived human macrophages treated with lipopolysaccharide (LPS) alone or in combination with CGS 21680 for two hours, and investigated the effects of A_{2A} receptors on expression of 1152 immune-related genes using custom nylon DNA microarrays. A detailed description of the experimental protocol and statistical analysis can be found in the Appendix.

Results and Discussion

Tables 4.1 and 4.2 can be found at the end of this Chapter and summarize data obtained from 4 experiments with human macrophages comparing gene expression between untreated cells vs. LPS, LPS vs. LPS + CGS 21680, LPS + CGS 21680 vs. LPS + CGS 21680 + ZM 241385, LPS vs. LPS + CGS 21680 + ZM 241385. Because the data presented in these tables are ratios of calculated Z scores, negative values indicate down-regulation and positive values indicate up-regulation of gene expression. Of particular interest are genes whose expression levels were up-regulated or down-regulated by treatement with CGS 21680 in combination with LPS as compared to LPS stimulation alone. Genes whose expression levels were down-regulated are shown in Table 4.1 and fall into distinct categories. CGS 21680 down-regulated the expression of a number of genes involved in transcription including GTF2H1 – general transcription factor IIH, LOC51082 – RNA polymerase I polypeptide D, USF2 – upstream transcription factor 2 that interacts with c-fos, CREB – cAMP response element binding protein, TPR – translocated promoter region, a protein involved in processing, intranuclear transport and targeting of RNA polymerase II transcripts, SNRPE – small nuclear ribonucleoprotein polypeptide E involved in RNA splicing, and TAF2G – RNA polymerase II, TATA box binding protein which initiates transcription by RNA polymerase II. Down-regulation of genes involved in transcription suggests that CGS 21680 may lead to interruption in gene expression by decreasing the expression of transcription factors and proteins involved in transcriptional initiation and transcript processing. Decreased mRNA

expression of TPR and SNRPE suggests interesting possibilities for potential post-transcriptional regulation of pro-inflammatory cytokines by A_{2A} receptors because these proteins are involved in RNA processing and transport, both important regulatory pathways for translation that we have not yet investigated for control of TNF-α protein expression.

CGS 21680 also down-regulated expression of genes involved in cell survival including CASP1 – caspase 1, APAF1 – apoptotic protease activating factor, MCL1 – myeloid cell leukemia sequence 1, an anti-apoptotic protein related to Bcl-2 and genes involved in development including PDGFB – PDGF β polypeptide, mitogenic for cells of mesenchymal origin, IGF2 – somatomedin A, HD – huntingtin, protein required for normal development, and PLK – polo-like kinase, protein involved in cyclin signaling and cell cycle regulation. In addition to being involved in signaling cell death, caspase 1 is a protease involved in processing and activating IL-1β. Down-regulation of caspase 1 suggests post-transcriptional regulation of cytokines by A_{2A} receptors involving protein processing by converting enzymes (ICE). Regulation of TNF-α converting enzyme (TACE) expression or activity may be an important target for adenosine receptors as a mechanism for regulation of TNF-α. Consistent with CGS 21680 affecting genes involved in cell survival are studies showing a role for A_3 receptors in mediating neuronal cell death (Abbracchio and Cattabeni, 1999).

Particularly interesting and relating to the role of adenosine in development is the finding that CGS 21680 down-regulated the expression of huntingtin because a role for adenosine receptors has been proposed in Huntington's disease (HD), a degenerative motor disorder resulting from tri-nucleotide repeats in the huntingtin gene. An A_1 receptor agonist was protective in an animal model of HD (Blum et al., 2002) and A_{2A} receptor expression was diminished in a transgenic mouse model of HD (Spektor et al., 2002). Low doses of the A_{2A} antagonist SCH 58261 were neuroprotective in an excitotoxic rat model of HD (Popoli et al., 2002) and the A_{2A} antagonist DMPX prevented EEG abnormalities in the same HD model (Reggio et al., 1999). Decreased huntingtin gene expression induced by CGS 21680 further supports work by other groups suggesting a potential role for adenosine receptors in HD although the role of huntingtin expression in macrophages and the role of macrophages in HD is unclear.

Findings particularly relevant for work presented in this thesis are that CGS 21680 also down-regulated the expression of several genes involved in cell signaling and immune regulation, including GADD45A – growth arrest and DNA-damage inducible α, a protein involved in regulating p38 and JNK signaling, MADD – MAPK activating death domain, a protein involved in TNFR1 signaling, CHRNA1 – nicotinic cholinergic receptor α polypeptide 1, a protein subunit involved in acetylcholine binding and channel gating, GNA15 – Gα15, part of the Gq family of G proteins, PLA2G5 – PLA2 group V, a protein involved in leukotriene synthesis, IL-9 – a cytokine that regulates hematopoietic cells by

activating STAT proteins, CCL20 – MIP-3α, a CC chemokine involved in immune regulation, NCF4 – neutrophil cytosolic factor 4, a protein involved in production of phagocytic oxygen radicals, ITGB1 - β1 integrin, a protein involved in cell adhesion, embryogenesis, hemostasis, tissue repair, immune function, and metastatic diffusion of tumour cells, ITGA4 - α4 integrin, a protein involved in T cell adhesion, fibronectin, thrombospondin, and VCAM-1 binding, and ITGAM - αM integrin, a protein involved in neutrophil and monocyte binding to stimulated endothelium and phagocytosis of complement coated particles. Of note for the effects of adenosine receptors on cell signaling pathways are down-regulated expression of GADD45, a protein involved in activating p38 and JNK signaling pathways via MKK4; of MADD, a protein involved in TNF-α signaling by TNFR1; and of Gα15 because this G protein has been shown to be coupled to A_{2A} receptors (Fredholm et al., 2001). This finding may help in understanding specific coupling of A_{2A} receptors to G protein subunits in immune cells and the immune modulatory mechanisms of A_{2A} receptors. Because ZM 241385 did not reverse the CGS 21680-induced down-regulation of Gα15 gene expression, the effects of CGS 21680 may be influenced by other adenosine receptors, by second messenger coupling of A_{2A} receptors and/or by regulating expression of G protein subunits. Down-regulation of CHRNA1 by CGS 21680, a subunit of nicotinic acetylcholine receptors, may help to explain studies showing decreased neuronal activity in cholinergic nuclei by adenosine (Dunwiddie and Masino, 2001). The finding that CGS 21680 down-regulated GADD45 expression may also help explain the transient nature of p38 dephosphorylation follwing treatment

with CGS 21680 but long-lasting inhibition of TNF-α protein expression. Adenosine A_{2A} receptor signaling may control TNF-α production initially by inactivating the p38 dependent signaling pathway whereas long-lasting TNF-α regulation involves down--egulating proteins involved in activating p38 and JNK signaling. Finally, CGS 21680 may regulate TNF-α not only by decreasing its protein expression, but also by decreasing expression of proteins involved in TNFR signaling like MADD, thereby blocking both expression and function of TNF-α.

Down-regulation of cytokine and chemokine gene expression by CGS 21680 suggests that A_{2A} receptors may regulate some immune proteins pre-transcriptionally. There are a limited number of studies published to date investigating the regulation of chemokines by adenosine. Several groups have shown increased production of IL-8 by mast cells induced by A_{2B} receptors (Feoktistov et al., 1999; Feoktistov et al., 2003; Meade et al., 2002). The adenosine receptor agonist NECA increased IL-8 production and release from human microvascular endothelial cells (Feoktistov et al., 2002). IL-8 acts primarily on neutrophils to stimulate activation and recruitment. Adenosine receptor activation increased CCL17 release from dendritic cells (Panther et al., 2003), decreased CXCL10 release from dendritic cells (Panther et al., 2003), and decreased MIP-1α production and release from macrophages (Szabo et al., 1998). MIP-1α acts on monocytes and T cells to stimulate activation and recruitment. CCL17 is involved in mediating T cell responses and CXCL10 is

involved in IFN-γ signaling. Our data suggest that in macrophages, A_{2A} receptor activation decreased expression of MIP-3α, which further implicates a role for these receptors in regulating inflammation and innate immunity by influencing lymphocyte activation and recruitment. Down-regulation of NCF4, a protein involved in phagocyte free radical production, compliments studies from Revan et al., who showed that A_{2A} receptor activation in neutrophils decreased free radical production and respiratory burst thereby disabling the ability of these cells to kill their targets (Revan et al., 1996).

Our findings that CGS 21680 down-regulated integrin expression, particularly α4 and β1 integrin, both of which bind fibronectin, emphasize the importance of our experiments investigating the effects of adenosine on monocyte adhesion. Further investigation of monocyte and T lymphocyte adhesion will help to clarify the role of adenosine and adenosine receptor activation in mediating tissue migration of immune cells, particularly in diseases like MS where immune cell migration into the CNS is a principal factor in disease pathology. Decreased A_1 receptor expression in blood and brain from MS patients (Johnston et al., 2001; Mayne et al., 1999), for example, may be associated with increased expression of integrins, which can contribute to increased recruitment of these cells into the CNS.

It is important to note that the A_{2A} receptor agonist ZM 241385 did not reverse any of the CGS 21680-induced decreases in gene expression. This suggests

that the effects of CGS 21680 on gene expression described so far were not mediated by adenosine A_{2A} receptors and implicates a role for A_1, A_{2B}, and/or A_3 receptors. Further studies with other selective adenosine receptor agonists and antagonists will be essential in differentiating the adenosine receptor specific actions on immune-related gene expression. Studies using adenosine receptor agonists and antagonists in the absence of inflammatory stimulus may also yield important information about affects on immune gene expression in unstimulated cells.

Finally, CGS 21680 down-regulated expression of other genes with less well defined connections to adenosine including AOC2 – amine oxidase, USP9X – ubiquitin specific protease 9, involved in ubiquitin removal and DDB1 – damage-specific DNA binding protein 1, involved in nucleotide-excision repair. Regulation of DDB1 suggests a potential role of adenosine for further regulation of DNA replication, in addition to regulation of transcriptional proteins. Modulation of signaling by ubiquitination is common for proteins like NF-κB and ATF-2, where activation of NF-κB involves ubiquitination of the inhibitor protein IκBα, and ubiquitination and degradation of ATF-2 controls its transcriptional activity. Hence, modulation of DDB1 and USP9X by adenosine may contribute to DNA replication, cell cycle regulation, and termination or activation of transcription factors.

The genes whose expression levels were up-regulated by CGS 21680 are listed in Table 4.2 and as with genes whose expression levels were decreased by CGS 21680 fall into distinct categories based on function. CGS 21680 treatment up-regulated genes involved in cell cycle and development including GSN – gelsolin, a protein involved in cell growth and apoptosis, MYBL2 – v-myb myeloblastosis viral oncogene homolog involved in cell cycle control, CCNH – cyclin H, a protein involved in regulating cell cycle progression, CCNA2 – cyclin A2, a protein involved in regulating cell cycle progression, E2F5 – E2F transcription factor 5, a protein involved in development, PRIM2A – primase polypeptide 2A, a protein involved in DNA replication, and BMI1 – B lymphoma Mo-MLV insertion region, a protein involved in regulating telomerase activity. CGS 21680 up-regulated genes involved in transcription including GTF2F2- general transcription factor IIf, NFIC – nuclear factor I/C, and TCEA1 – transcription elongation factor A and in cell survival including PDGFRL – PDGF receptor, a protein involved in growth factor signaling that acts as a tumour suppressor, and END1 – endothelin 1, a protein with anti-apoptotic actions. These data suggest adenosine receptors may play an important role in regulating expression of proteins that control the cell cycle because expression of the cell cycle regulators E2F5 and MYBL2 were increased by CGS 21680. Cyclin H expression was increased by CGS 21680 and this protein regulates CDK7 and CDK2, and forms a component of TFIIH and RNA polymerase II protein complexes. This suggests that by increasing cyclin H expression, CGS 21680 may regulate a link between basal transcriptional activity (TFIIH, RNA polymerase II) and cell cycle machinery (CDKs). In addition, CGS

21680 may also play a role in regulating DNA replication and cell growth by influencing expression of primase, BMI1, a protein involved in inducing telomerase activity, and gelsolin, a protein that regulates cell growth and apoptosis. These data, together with data showing down-regulation of other genes involved in development and transcription, suggest long-lasting control of fundamental cellular processes by adenosine and adenosine receptors by potentially influencing expression of proteins described above.

Treatment with CGS 21680 up-regulated genes of proteins involved in modulating immune responses and those included SYCA19 – chemokine MIP-3β, a protein involved in normal lymphocyte recirculation and homing that binds to CCR7, BLR2 – chemokine (CC) receptor 7, a protein involved in migration of chronic lymphocytic leukemia cells into lymph nodes, GRO1 – chemokine (CXC) ligand 1, a protein involved in angiogenesis, chemoattraction and activation of neutrophils, IL2 – IL-2 is involved in proliferation of lymphocytes, and TCF12 – transcription factor 12 involved in regulating gene expression in lymphocytes. These data suggest possible up-regulation of chemokine signaling resulting from adenosine receptor activation because CGS 21680 increased expression of genes encoding both MIP-3β and its receptor CCR7. This signaling pathway is normally involved in trafficking and migration of lymphocytes and studies of protein expression will help determine whether increased mRNA expression by CGS 21680 is a compensatory mechanism resulting from decreased chemokine activity or whether adenosine receptors positively regulate this chemokine

pathway. However, little is known about chemokine regulation by adenosine and how these data may fit into the immune modulatory theory for adenosine. CGS 21680 up-regulated gene expression of the cytokine IL-2 and other groups have shown that adenosine, acting primarily via A_2 receptors, decreased production of IL-2 protein, the primary growth factor for T lymphocytes (Hoskin et al., 2002). Regulation of IL-2 by adenosine may be cell-type specific, however increased IL-2 gene expression may also be a compensatory mechanism of the cell to override inhibitory actions of CGS 21680 on IL-2 protein production and/or release.

CGS 21680 increased expression of genes involved in cell signaling including MAPK10 – neuronal JNK, RPS6KA3 – p90S6K, a protein involved in MAPK signaling, FGR – SRC2, a tyrosine kinase family member, SRC – SRC1, a tyrosine kinase family member, PIK3R1 – PI3K p85α, a regulatory subunit of PI3K, and NYP1R – neuropeptide Y receptor Y1, a protein signaling neuropeptide Y whose effects are mediated through CaM kinase and CREB. As with the CGS 21680-mediated up-regulation of genes involved in immune regulation, the function of increased expression of cell signaling genes is unclear and could result both from positive actions of adenosine on these pathways or from a compensatory effort of the cell to override inhibitory actions of adenosine. Functional studies of protein expression will help clarify the roles of these signaling molecules in adenosine receptor signaling. Interestingly, CGS 21680 up-regulated expression of 2 genes involved in MAPK signaling, although the

function of neuronal JNK and p90S6K in macrophages is not known, neuronal JNK is implicated in mediating apoptosis of neurons (Marques et al., 2003). In addition, pharmacological studies showed that PI3K inhibition by wortmannin partially decreased PMA/PHA-induced TNF-α production in U937 cells and DNA microarray data showed that CGS 21680 upregulated PI3K p85α subunit expression, which is a ubiquitously expressed subunit of PI3K that regulates subcellular localization, activity and stability of enzyme activity. Finally, CGS 21680 up-regulated SRC1 and SRC2 gene expression. Both proteins are involved in tyrosine kinase signaling and SRC1 regulates cell adhesion by selectins, integrins, and adhesion molecules like ICAM-1. The effect of CGS 21680 on SRC1 protein expression or activity is unknown, however given our preliminary data on cell adhesion and integrin mRNA expression mediated by CGS 21680, this pathway may be an important area of investigation to further understand the role of adenosine in regulating cell adhesion.

The up-regulation of several immune genes by CGS 21680 was blocked by ZM 241385 and those data suggest that up-regulation of these genes by CGS 21680 was A_{2A} receptor mediated (Table 4.3). As previously mentioned, further studies with receptor-selective agonists will help clarify the effects of specific adenosine receptor actions on specific genes.

In summary, data presented from microarray studies have identified interesting and important areas of further study towards understanding immune regulation

by adenosine receptors. Adenosine and adenosine receptors may mediate integrin expression and consequently affect cell adhesion. p38 and JNK signaling pathways may be affected through regulation of GADD45 expression following A_{2A} receptor activation. Chemokine and cytokine function may be mediated through down-regulation of IL-9 and MIP-3α expression and upregulation of IL-2, MIP-3β, and CCR7 expression following A_{2A} receptor activation. Further studies examining the role of adenosine and adenosine receptors in mediating chemokine gene expression, protein production, and biological function will contribute substantially to our understanding of endogenous control of immune responses.

GENE	L+C vs. L		L+C+ZM vs. L+C		L+C+ZM vs. L		L vs. untx		PROTEIN
	Z	P	Z	P	Z	P	Z	P	
AOC2	-2.01	0.045	-2.19	0.028	-0.26	0.796	1.24	0.216	amine oxidase, copper containing 2
CASP1	-2.01	0.044	-3.80	0.000	0.31	0.754	0.92	0.359	caspase 1 (IL-1 beta convertase)
PLA2G5	-2.05	0.040	-4.31	0.000	2.47	0.014	3.30	0.001	PLA2
GTF2H1	-2.06	0.040	-2.49	0.013	-0.31	0.754	0.45	0.654	general transcription factor IIH
SCYA20	-2.09	0.036	0.14	0.888	0.81	0.419	-1.96	0.050	MIP-3a
APAF1	-2.11	0.035	-0.28	0.778	0.37	0.712	1.09	0.275	apoptotic protease activating factor
LOC51082	-2.12	0.034	-2.01	0.045	-0.40	0.688	0.55	0.580	RNA polymerase I polypeptide D
USF2	-2.16	0.030	-1.56	0.120	0.53	0.596	-0.73	0.468	upstream transcription factor 2
GNA15	-2.19	0.028	-2.52	0.012	2.65	0.008	0.54	0.590	G protein, alpha 15 (Gq class)
CREBBP	-2.21	0.027	-2.21	0.027	2.44	0.015	1.45	0.147	CREB
CHRNA1	-2.21	0.027	-3.76	0.000	-1.21	0.228	2.30	0.022	cholinergic receptor, nicotinic, alpha polypeptide 1
MCL1	-2.23	0.026	0.79	0.430	3.91	0.000	0.70	0.484	myeloid cell leukemia sequence 1
TPR	-2.25	0.024	-3.42	0.001	1.45	0.148	1.60	0.109	translocated promoter region
IL9	-2.26	0.024	-2.06	0.039	-2.02	0.044	-0.52	0.604	IL-9
NCF4	-2.28	0.022	-3.55	0.000	2.15	0.031	1.26	0.208	neutrophil cytosolic factor 4
ITGAM	-2.32	0.020	-1.85	0.065	1.86	0.063	3.19	0.001	integrin, alpha M
USP9X	-2.33	0.020	-1.61	0.107	-0.06	0.952	1.14	0.252	ubiquitin specific protease 9
SNRPE	-2.35	0.019	-2.81	0.005	-0.24	0.809	0.73	0.463	small nuclear ribonucleoprotein polypeptide E
DDB1	-2.48	0.013	-3.66	0.000	-2.65	0.008	2.97	0.003	damage-specific DNA binding protein 1
ITGA4	-2.54	0.011	-3.77	0.000	-0.51	0.610	1.26	0.207	integrin, alpha 4
PDGFB	-2.57	0.010	-0.06	0.950	0.40	0.690	-0.69	0.492	PDGF beta polypeptide
IGF2	-2.75	0.006	-0.19	0.853	-1.26	0.208	-1.09	0.274	IGF-2
MADD	-2.85	0.004	-0.44	0.662	-17.81	0.000	-1.14	0.256	MAPK-activating death domain
HD	-2.93	0.003	-1.66	0.097	7.23	0.000	1.86	0.062	huntingtin
TAF2G	-2.99	0.003	0.02	0.983	1.32	0.187	2.26	0.024	RNA polymerase II, TATA box binding protein
PLK	-3.11	0.002	-0.83	0.404	3.11	0.002	0.53	0.597	polo-like kinase
ITGB1	-3.26	0.001	-0.66	0.509	0.45	0.655	-0.46	0.647	integrin, beta 1
GADD45A	-3.36	0.001	-5.04	0.000	-0.28	0.778	1.56	0.120	growth arrest and DNA-damage-inducible, alpha

Table 4.1: Down-regulation of gene expression by CGS 21680. Primary human macrophages were pre-treated with CGS 21680 (C; 1 μM) alone or in combination with ZM 241385 (ZM; 100 nM) for 30 minutes prior to stimulation with LPS (L; 100 ng/ml). Following LPS treatment for 2 hours, cells were collected, and gene expression was determined by DNA microarray analysis. Z – ratio of Z scores, P – two tailed t-test p value

GENE	L+C vs. L		L+C+ZM vs. L+C		L+C+ZM vs. L		L vs. untx		PROTEIN
	Z	P	Z	P	Z	P	Z	P	
PDGFRL	5.49	0.000	0.01	0.993	0.48	0.633	-2.87	0.004	PDGF receptor-like
EDN1	3.00	0.003	0.52	0.600	0.87	0.386	-0.31	0.756	endothelin 1
GSN	2.99	0.003	1.52	0.130	0.98	0.327	-2.07	0.038	gelsolin
SCYA19	2.97	0.003	-5.04	0.000	-3.46	0.001	-1.52	0.130	MIP-3b
MAPK10	2.75	0.006	6.33	0.000	1.68	0.094	-1.51	0.131	MAPK10
GTF2F2	2.74	0.006	3.15	0.002	13.83	0.000	0.90	0.370	general transcription factor IIF, polypeptide 2
GFAP	2.72	0.007	0.27	0.788	0.77	0.440	-0.82	0.413	GFAP
MYBL2	2.69	0.007	-1.54	0.124	-0.50	0.614	-3.26	0.001	v-myb myeloblastosis viral oncogene homolog
CCNH	2.50	0.012	-2.80	0.005	-1.86	0.063	-1.68	0.093	cyclin H
CCNA2	2.46	0.014	0.68	0.496	0.81	0.418	-2.82	0.005	cyclin A2
CCR7	2.43	0.015	0.39	0.695	-0.65	0.514	0.08	0.937	chemokine (CC motif) receptor 7
E2F5	2.41	0.016	-7.57	0.000	-4.88	0.000	-1.50	0.134	E2F transcription factor-5
IL2	2.37	0.018	4.67	0.000	18.90	0.000	-0.59	0.555	IL-2
NFIC	2.35	0.019	-2.13	0.033	2.45	0.014	-0.81	0.421	nuclear factor I/C
SACM2L	2.30	0.022	-4.51	0.000	-0.18	0.858	-1.21	0.225	SAC2 suppressor of actin mutations 2-like
SRC	2.29	0.022	0.60	0.546	-0.18	0.854	-2.19	0.028	v-scr sarcoma viral ongogene homolog
GRO1	2.23	0.026	3.70	0.000	3.95	0.000	-0.56	0.573	chemokine (CXC motif) ligand 1
NPY1R	2.20	0.028	-3.36	0.001	1.21	0.227	-0.34	0.735	neuropeptide Y receptor Y1
FGR	2.17	0.030	0.23	0.816	1.26	0.208	-3.17	0.002	Gardner-Rasheed feline sarcoma viral oncogene
PIK3R1	2.15	0.031	-1.65	0.099	0.41	0.682	-1.37	0.171	PI3K, regulatory subunit p85 alpha
BMI1	2.14	0.032	-1.85	0.064	0.43	0.665	0.64	0.519	B lymphoma Mo-MLV insertion region
RPS6KA3	2.09	0.037	-2.93	0.003	-0.28	0.783	-0.22	0.828	ribosomal protein S6 kinase, p90S6k
PRIM2A	2.09	0.037	0.79	0.427	0.27	0.784	-0.50	0.621	primase, polypeptide 2A
GABPB2	2.03	0.042	-1.84	0.066	1.71	0.087	-0.99	0.324	GA binding protein transcription factor, beta subunit 2
TCEA1	2.02	0.043	0.36	0.722	1.05	0.292	-0.51	0.612	transcription elongation factor A
TCF12	2.00	0.045	-2.43	0.015	-3.09	0.002	-3.42	0.001	transcrption factor I2

Table 4.2: Up-regulation of immune gene expression by CGS 21680. Primary human macrophages were pre-treated with CGS 21680 (C; 1 μM) alone or in combination with ZM 241385 (ZM; 100 nM) for 30 minutes prior to stimulation with LPS (L; 100 ng/ml). Following LPS treatment for 2 hours, cells were collected, and gene expression was determined by DNA microarray analysis. Z – ratio of Z scores, P – two tailed t-test p value

	L+C vs. L		L+C+ZM vs. L+C		
	Z	P	Z	P	
SCYA19	2.97	0.003	-5.04	0.000	chemokine ligand 19 (CC motif)
MYBL2	2.69	0.007	-1.54	0.124	v-myb myeloblastosis viral oncogene homolog
CCNH	2.50	0.012	-2.80	0.005	cyclin H
E2F5	2.41	0.016	-7.57	0.000	E2F transcription factor 5
NFIC	2.35	0.019	-2.13	0.033	nuclear factor I/C
SACM2L	2.30	0.022	-4.51	0.000	SAC2 suppressor of actin mutations 2
NPY1R	2.20	0.028	-3.36	0.001	neuropeptide Y receptor Y1
PIK3R1	2.15	0.031	-1.65	0.099	PI3K regulatory subunit p85alpha
BMI1	2.14	0.032	-1.85	0.064	B lymphoma Mo-MLV insertion region
RPS6KA3	2.09	0.037	-2.93	0.003	ribsosomal protein S6 kinase (p90S6k)
GABPB2	2.03	0.042	-1.84	0.066	GA binding protein transcription factor
TCF12	2.00	0.045	-2.43	0.015	transcription factor 12

Table 4.3: Up-regulation of immune gene expression by CGS 21680. Primary human macrophages were pre-treated with CGS 21680 (C; 1 μM) alone or in combination with ZM 241385 (ZM; 100 nM) for 30 minutes prior to stimulation with LPS (L; 100 ng/ml). Following LPS treatment for 2 hours, cells were collected, and gene expression was determined by DNA microarray analysis. Z – ratio of Z scores, P – two tailed t-test p value

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Future Directions

Further exploring the cell signaling mechanisms activated by A_{2A} receptors that mediate pro-inflammatory cytokine production can make important contributions towards our understanding of how and why adenosine mediates immune responses. In particular, identifying the phosphatase pathways activated by A_{2A} receptors will not only broaden our understanding of TNF-α regulation, but also regulation of other important cell signaling pathways by adenosine receptors. Furthermore, the adenosine-activated phosphatase pathway may regulate several biological functions of a cell and/or tissue in addition to pro-inflammatory cytokine production.

Our data suggest that the anti-TNF-α effects of adenosine A_{2A} receptors are mediated by serine/threonine protein phosphatase activity, however the expression of specific phosphatases is unknown in macrophages or monocytes. As a result, further study to identify the specific regulatory subunits expressed and activated in these cells using RT-PCR and western blots will help identify the specific pathways activated by adenosine A_{2A} receptors. Our data suggest that PP1 and PP2A may be involved in mediating the anti-TNF-α effects of CGS 21680. As a result, a first step would be to identify the catalytic subunit isoforms and regulatory subunit isoforms of PP1 and PP2A expressed in U937 cells. Using the commercially available serine/threonine phosphatase activity kit and specific inhibitors of phosphatases like PP1 and PP2A, the contribution of individual phosphatase enzymes to CGS 21680 activated phosphatase activity will be identified. An important pathway to study in addition to PP1 and PP2A is

PP2Cα-2, a serine/threonine protein phosphatase shown to inactivate p38 in vivo and in vitro (Tamura et al., 2002). In addition, the anti-TNF-α effects of CGS 21680 were partially reversed by sodium orthovanadate, a broad-spectrum tyrosine phosphatase inhibitor. Using a commercially available tyrosine phosphatase enzyme activity kit, identification of tyrosine phosphatase activity induced by CGS 21680 using similar protocols as outlined above for serine/threonine phosphatases help determine the importance of these pathways in A_{2A} receptor signaling and if they contribute to cytokine regulation. Our initial experiments would focus on HePTP and LC-PTP. Both are tyrosine phosphatases expressed in hematopoietic cells and target p38 and ERK (Tamura et al., 2002). Finally, recent studies have identified dual specificity phosphatases (MKPs) in macrophages that regulate both MAPK signaling pathways and pro-inflammatory cytokine production (Chen et al., 2002). Because adenosine A_{2A} receptors may activate both serine/threonine and tyrosine phosphatase activity, both phosphatase specificities may contribute to down regulating TNF-α production, and because several proteins in the MAPK signaling pathways are phosphorylated on both serine/threonine and tyrosine residues, it is important to determine whether these newly identified molecules are activated by adenosine A_{2A} receptors. Because specific pharmacological inhibitors of these pathways are not available, studies targeting dual specificity phosphatases would involve detection of MKP mRNA and protein expression induced by CGS 21680. Our initial studies would focus on expression of MKP-1 because this protein has been implicated as a negative regulator of pro-

inflammatory cytokine production in macrophages and preferentially dephosphorylates JNK and p38 (Chen et al., 2002). In addition, MKP-4 and MKP-5 dephosphorylate JNK and p38 and are localized to nucleus and cytoplasm. We detected increased serine/threonine phosphatase activity in cytosolic preparations from cells treated with CGS 21680; therefore MKP-4 and MKP-5 are important pathways to study with respect to A_{2A} receptor activation (Tamura et al., 2002).

To date, our experiments have focused on adenosine A_{2A} receptors using pharmacological agents. In order to more conclusively identify A_{2A} receptor mediated signaling pathways, future experiments should be conducted using adenosine and adenosine A_{2A} receptor agonists in monocytes and macrophages isolated from A_{2A} receptor deficient mice. The experiments are particularly important given that changes in expression of some immune genes and in CGS 21680-induced serine/threonine phosphatase activity were not reversed by an A_{2A} receptor selective antagonist. Experiments to investigating p38 activity and TNF-α mRNA stability using macrophage cultures from A_{2A} receptor deficient mice will also help identify the role of these factors in mediating the anti-TNF-α effects of A_{2A} receptor activation. In addition, if adenosine A_{2A} receptors are involved in normal control of immune responses and pro-inflammatory cytokine production, higher levels of TNF-α following inflammatory stimulus would be expected as well as increased half-life and stability of TNF-α mRNA in mice deficient in A_{2A} receptors.

The theory has been proposed that adenosine is an endogenous regulator of immune responses. To focus our interest on the anti-inflammatory effects of adenosine A_{2A} receptors towards more big-picture implications of these signaling pathways, experiments can be designed to investigate the effects of A_{2A} receptors on mRNA stability of other transiently expressed proteins. Our proposal, that A_{2A} receptors decrease TNF-α mRNA stability by dephosphorylating p38 and targeting a pathway that stabilizes mRNA via 3'UTR AREs, is not necessarily specific for TNF-α. In fact, AREs regulate several transiently expressed proteins involved in immune responses. As a result, experiments designed to identify the effects of CGS 21680 on cytokines and chemokines simultaneously using RNase protection assays will identify whether the TNF-α effects of A_{2A} receptors are specific for TNF-α or whether A_{2A} receptors regulate immune proteins in general by decreasing mRNA stability.

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General Discussion

The adenosine system has been an elusive target of pharmaceutical intervention for many years. Adenosine itself has been used clinically and adenosine receptor agonists have been tested in clinical trials. Currently, adenosine is used to restore normal heart rhythm in paroxysmal supraventricular tachycardia and as an adjunct for thallium cardiac imaging to evaluate coronary artery disease (Fredholm et al., 2001). Adenosine A_1 antagonists have been used in clinical trials for the treatment of acute renal failure in patients with congestive heart failure and adenosine A_{2A} receptor antagonists have been tested clinically for the treatment of Parkinson's disease (Fredholm et al., 2001). In addition, the actions of some clinically used agents may be mediated through adenosine and adenosine receptors. The mechanism of action of methotrexate, a drug used to treat rheumatoid arthritis, involves increasing adenosine levels (Montensinos et al., 2003). Theophylline, a drug used to treat asthma, may act by antagonizing adenosine receptors in the lung (Fredholm et al., 2001).

Recently it was proposed that adenosine acts as a "metabolic switch" capable of sensing different degrees of inflammation and tissue damage and subsequently triggering the appropriate immune response (Sitkovsky, 2003). This proposal was based on findings that adenosine levels are increased during inflammatory responses and following tissue damage in a manner that reflects the severity of the insult or inflammation. In fact, studies in the heart suggest that increased adenosine levels accompanying local inflammation may result from hypoxia and hypoxia-induced inhibition of adenosine kinase to cause increased endogenous

adenosine levels. In the heart, experimental data indicates that 80% of adenosine metabolism is normally via AK, so inhibition of this pathway during hypoxia would significantly increase local adenosine levels (Sitkovsky, 2003).

The ultimate effects of adenosine can be beneficial or harmful depending on receptor expression, cell signaling pathways activated by adenosine receptors, local adenosine concentrations, and the conditions studied (Blackburn, 2003). For example, mice deficient in adenosine A_{2A} receptors show increased tissue damage and cytokine production resulting from doses of inflammatory stimuli that do not induce disease in wild type mice. Wild type mice injected with the adenosine A_{2A} receptor agonist ZM 241385 also developed tissue damage and cytokine production resulting from sub-threshold doses of inflammatory stimuli (Blackburn, 2003). These studies suggest a beneficial and anti-inflammatory role for activation of adenosine A_{2A} receptors. A_{2A} receptor activation is also protective in ischemia-reperfusion injury of kidney, skin, lung, blood vessels, brain, and spinal cord (Mayne et al., 2001; Sitkovsky, 2003).

Whereas A_{2A} receptors have been shown to regulate immune responses by inhibiting neutrophil activation and pro-inflammatory cytokine production, the roles of A_1 and A_3 receptors are less clearly defined. Although both A_1 and A_3 receptors can inhibit pro-inflammatory cytokine production, they can also mediate pro-inflammatory effects. Specifically, A_1 receptors promote chemotaxis of neutrophils and adherence of neutrophils to endothelium and A_3 receptors

promote mast cell degranulation (Sitkovsky, 2003). Complicating the picture are in vivo studies showing that mice deficient in ADA develop severe combined immunodeficiency (SCID) and these mice exhibit increased extracellular adenosine levels that can reach 100 μM in the lung, more than 100 fold higher than normal levels (Adanin et al., 2002; Blackburn, 2003). ADA deficient mice develop pulmonary inflammation and airway remodeling accompanied by mast cell degranulation, increased macrophages and eosinophils in the lung, airway enlargement and hyperresponsiveness and several of these symptoms are improved when mice are given ADA therapy to decrease adenosine levels. In fact, ADA deficient mice which typically die at 3 weeks of age from respiratory failure survive longer with ADA (Adanin et al., 2002; Blackburn, 2003).

The seemingly contradictory results showing both possible pro- and anti-inflammatory actions of adenosine highlight the complexity of this system as well as the importance of studying adenosine A_{2A} receptors in particular. Whereas A_1 and A_3 receptors may be both pro- and anti-inflammatory depending on the model system used, studies with A_{2A} receptors have consistently identified anti-inflammatory properties of this receptor. Data from A_{2A} receptor deficient mice also suggest a non-redundancy in the anti-inflammatory actions of A_{2A} receptors (Fredholm et al., 2001; Sitkovsky, 2003), further emphasizing their importance in controlling immune responses. Studies designed to elucidate the anti-inflammatory properties of adenosine A_{2A} receptors will contribute to our

understanding of endogenous control of immune responses and highlight potential avenues for therapeutic intervention against inflammatory disorders.

Our studies were designed to identify cell signaling mechanisms mediating the anti-TNF-α effects of adenosine A_{2A} receptors. Studying specific adenosine receptor-mediated signaling mechanisms in vivo is difficult because appropriate animal models are not readily available. ADA deficient mice are not suitable for studies aimed at specific adenosine receptor mediated signaling mechanisms because high endogenous adenosine levels activate all 4 receptors. A_{2A} receptor deficient mice are not readily available. Ideally, studies in mice, which could be induced to down-regulate A_{2A} receptor expression using an inducible expression system such as the lac operon would nicely, identify A_{2A} receptor selective effects on immune responses. This technology is not available in our laboratory and consequently we conducted in vitro studies using a model well established in our laboratory to study A_{2A} receptor activation in PMA/PHA-stimulated promonocytic U937 cells. Based on the literature described in Chapter 3, the cellular mechanisms mediating the anti-TNF-α effects of adenosine receptors in phagocytes may be differentiation state specific, meaning that the signaling mechanisms differ for monocytes and macrophages. As discussed earlier in the individual chapters, our data suggest that the mechanisms we identified in U937 cells resemble those identified by others in both established macrophage cell lines and primary macrophage cultures. We confirmed, in our experimental model, data published by others in macrophages

(Findings 1, 2, 3, and 4) and neutrophils (Finding 5). Specifically, (1) adenosine receptor agonists inhibit TNF-α protein production, (2) adenosine A_{2A} receptor activation inhibits TNF-α production in a manner independent of cAMP/PKA signaling pathways, (3) A_{2A} receptor occupancy inhibits TNF-α protein production without inhibiting transcription factor activity or induction of TNF-α gene expression, (4) A_{2A} receptor activation does not decrease activity of the ERK signaling pathway, and (5) adenosine A_{2A} receptor activation increases serine/threonine phosphatase activity. We also identified several novel signaling mechanisms mediating the anti-TNF-α actions of adenosine A_{2A} receptors. Specifically, (1) adenosine A_{2A} receptor activation decreases stability and half-life of TNF-α mRNA, (2) adenosine A_{2A} receptor activation increases cytosolic serine/threonine phosphatase activity, (3) the anti-TNF-α actions of A_{2A} receptors require protein phosphatase activity, and (4) A_{2A} receptor activation decreases basal activity of the MAPK p38. Putting our data together, we propose a model for adenosine A_{2A} receptor mediated inhibition of TNF-α production (Figure 1). Occupancy of adenosine A_{2A} receptors activates one or multiple protein phosphatases, which decrease basal activity of p38 by dephosphorylating p38 directly or indirectly by dephosphorylating an upstream activator of p38 like MKK6. Dephosphorylation of p38 decreases activity of a p38 dependent signaling pathway, which normally stabilizes TNF-α mRNA via 3' UTR AREs, possibly through a protein like TTP (Mahtani et al., 2001), resulting in increased degradation and decreased half-life of TNF-α mRNA. Increased mRNA

degradation by A_{2A} receptor activation impedes protein production of TNF-α that is otherwise induced by PMA/PHA.

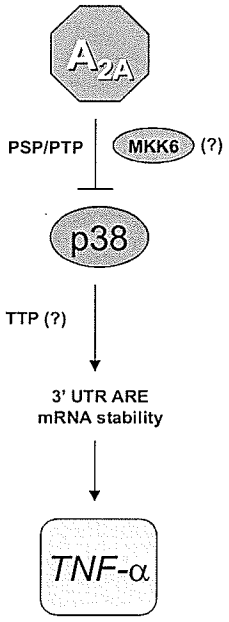

Figure 1: Signaling Model of Adenosine A_{2A} Receptor Activation. Adenosine A_{2A} receptor activation increases PSP and or PTP to decrease phosphorylation of p38 either directly or indirectly by decreasing activity of an upstream activator like MKK6. Inhibition of the p38 signaling pathway decreases stability of mRNA through 3'UTR AREs. PSP – serine/threonine phosphatase, PTP – tyrosine phosphatase

Our results demonstrating that adenosine A_{2A} receptor occupancy decreases TNF-α protein production post-transcriptionally is therapeutically important primarily because it increases the window of opportunity for pharmacological interventions targeted at A_{2A} receptors. Specifically, inhibition of TNF-α protein production by A_{2A} receptors did not require pre-treatment with the A_{2A} agonist and was statistically significant when the A_{2A} agonist was added up to 1 hour following inflammatory challenge.

Recent findings suggest that expression of adenosine A_{2A} receptors is dependent on both activation of and the differentiation state of cells (Khoa et al., 2001). This study found that expression of adenosine A_{2A} receptors was increased during macrophage differentiation and following treatment of THP-1 cells with pro-inflammatory cytokines suggesting that A_{2A} receptor expression increases when monocytes are either induced to differentiate or exposed to inflammatory stimuli. Activity and differentiation dependent expression of adenosine A_{2A} receptors further supports the potential for targeting these receptors therapeutically by widening the window of opportunity for intervention.

Our observed anti-TNF-α effects of CGS 21680 were long lasting, with statistically significant inhibition of TNF-α following 8 hours of stimulation with PMA/PHA. Our signaling model proposes that A_{2A} receptors activate a protein phosphatase that decreases phospho-p38 protein levels and decreases TNF-α protein production by destabilizing TNF-α mRNA. Although the changes in serine/threonine phosphatase activity and p38 phosphorylation by CGS 21680 were rapid and transient, downstream effects of these proteins were longer lasting as demonstrated by inhibition of TNF-α protein production for up to 8 hours following inflammatory stimulus. The kinetics of activation and inactivation of effectors downstream of p38 that regulate mRNA stability may be slower than for the serine/threonine phosphatase activated by A_{2A} receptors and for dephosphorylation of p38. This would account for long lasting effects from short,

transient phosphatase activation and p38 dephosphorylation induced by CGS 21680. In addition, p38 itself or downstream effectors of p38 may physically associate with other proteins whose function depends on p38 phosphorylation. Activity of some proteins, like ATF-2 and IκBα discussed earlier, is controlled by ubiquitination (Fuchs et al., 2000; Ghosh and Karin, 2002). It is also possible that proteins whose activity depends on association with and/or phosphorylation of p38 are targeted for ubiquitination following dephosphorylation of p38 by CGS 21680. Degradation of proteins whose activity is regulated by p38 would require sufficient time to recover activity of these pathways, leading to a long lasting effect of adenosine A_{2A} receptor activation.

Data from our microarray studies however open the possibility of an alternative scenario. Treatment of human macrophages with CGS 21680 in the presence of LPS decreased mRNA expression of GADD45, a protein involved in activating p38 and JNK signaling pathways by regulating MKK4 and MKK6, proteins that function upstream of p38 and JNK (Lu et al., 2001). From these data, we hypothesize that initial dephosphorylation of p38 immediately shuts down the p38 signaling pathway involved in stabilizing TNF-α mRNA. Although the initial decrease in phospho-p38 persists for 5-15 minutes, downstream effectors of p38 may be affected for longer durations of time as discussed above. While increases in phosphatase activity may immediately decrease p38 signaling, long lasting effects of adenosine A_{2A} receptor activation may result from down-regulation of GADD45. In this case, initial decreases in phospho-p38 levels are

evident at 5-10 minutes following CGS 21680 treatment and more sustained decreases in phospho-p38 levels would be evident at time points coinciding with decreased GADD45 expression. Further studies will determine the effect of CGS 21680 on GADD45 mRNA and protein expression and phosphorylation of p38 and p38 activators at longer time intervals.

Although several studies have proposed that adenosine functions as an endogenous modulator of immune function (Mayne et al., 2001; Sitkovsky, 2003), and potentially as a sensor for levels of tissue damage and inflammation, our work and that by others has focused on individual adenosine receptors or regulation of single cell types, both small pieces of the adenosine puzzle. Therefore, it is important to put our findings in context with the literature and attempt to draw a big picture of how adenosine modulates immune responses. The general introduction details the known effects of adenosine on immune cells and proteins that mediate immune responses and are outlined here in Table 1. These effects, in general terms, seem to suggest that adenosine production associated with inflammation, tissue damage, or infection would favour a humoral, TH2 type immune response dominated by B cell activation and by cytokines like IL-4, IL-5, and IL-10.

	A_1	A_{2A}	A_{2B}	A_3
Affinity	Ki ≈ 70 nM	Ki ≈ 150 nM	Ki ≈ 5100 nM	Ki ≈ 6500 nM
Activation	low ado	low ado	high ado	high ado
Macrophages	↓TNF-α ↑ IL-10	↓ TNF-α , IL-12 ↑ IL-10		↓TNF-α ↑ IL-10
Neutrophils		↓ free radicals (A_2) ↓ respiratory burst (A_2) ↓ adhesion (A_2)		
Dendritic Cells	↑ chemotaxis	↑ maturation (A_2) ↑ activity (A_2) ↑ Ag presentation by MHCI (A_2)		↑ chemotaxis
T cells			↓ IL-2	↓ proliferation ↓ CTL activity ↓ IL-2, IFN-γ
Mast Cells	↑ degranulation		↑ degranulation	↑ degranulation

Table 1: Summary of adenosine effects on immune cells. Effects of A_2 receptors on neutrophils and dendritic cells may be A_{2A} and/or A_{2B}. The specific adenosine receptors that decrease α4β7 integrin-mediated adhesion of T lymphocytes are unknown. The specific adenosine receptors that increase IL-6 and IL-8 are also unknown.

In context of the hypothesis that adenosine acts as a sensor of inflammation, the effects of specific adenosine receptors on immune cells becomes important with respect to local adenosine concentration and affinity of each receptor for its ligand. In acting as a sensor or indicator of inflammation, high levels of adenosine would be associated with more severe inflammation or tissue damage and would activate both high and low affinity adenosine receptors. A_{2B} and A_3 receptor activation is associated with decreased T lymphocyte proliferation, decreased CTL activity, decreased IL-2 production, and decreased IFN-γ production (Hoskin et al., 2002; Huang et al., 1997; Mirabet et al., 1999). These

effects appear to decrease the initiation of adaptive immunity. In addition, A_{2B} and A_3 receptor activation is associated with increased mast cell degranulation (Fredholm et al., 2001; Klinger et al., 2002). These effects may be critical for the role adenosine plays in immune responses because macrophages and mast cells already stationed in the tissue mediate the initiation of the response to either infection or tissue injury (Nathan, 2002).

Mast cells respond by causing vasodilation and extravasation of fluid, and by activating sensory nerve endings, endothelium, and neutrophils, ultimately leading to leukocyte recruitment (Nathan, 2002). Although it seems contradictory that adenosine would increase mast cell responses, given its other effects, it is important to point out that the initiation of inflammation provides information that determines the type of injury and subsequent response. For example, if the initial trigger event is tissue damage, ensuing inflammation must detect if there is an accompanying infection that requires an immune response. If infection initiates the response, there must be a determination about possible tissue injury. When both events co-exist, termination of infection is the primary goal (Nathan, 2002). Due to the nature of the immune system and its capacity for destruction, the ability of an initial inflammatory response to sense and direct an appropriate immune response defends against infection and protects healthy tissue. As initial mediators of inflammation, mast cells are essential in assisting with the detection of infection and tissue damage (Nathan, 2002). As a result, activation of this process during events producing high adenosine levels suggests that

adenosine may be an important player in the initial determination of how the immune system needs to respond. This fits nicely with our proposal that adenosine guides or modulates a type of immune response.

Macrophages as tissue-resident immune cells also function as initial mediators of inflammation. Because A_1, A_{2A}, and A_3 receptors decrease pro-inflammatory cytokine production by macrophages (Bouma et al., 1994; Bshesh et al., 2002; Hasko et al., 2000; Hasko et al., 1996; Link et al., 2000; Mayne et al., 2001; Ohta and Sitkovsky, 2001; Sitkovsky, 2003), adenosine may serve initially to activate the essential protective process but also to temper the ensuing response. It may be that by decreasing pro-inflammatory cytokine production by macrophages following the initial insult, adenosine helps protect local tissue while the type of required immune response is determined. We propose that adenosine acts initially as a sensor and regulator of local inflammatory responses to initiate mast cell degranulation by engaging low affinity receptors during extreme tissue damage or inflammation and by protecting local tissue through control of pro-inflammatory cytokine production by tissue-resident macrophages by engaging both low and high affinity receptors.

Following activation of mast cell degranulation, leukocytes, particularly neutrophils and T lymphocytes, are recruited to the inflammatory site (Nathan, 2002). Adenosine likely assists in modulating the local immune response by decreasing proliferation and activation of T lymphocytes. Acting on T

lymphocytes via A_3 receptors, effects of adenosine on T cell responses are limited to areas where adenosine levels are high, suggesting that adenosine modulates activation of adaptive immune responses during severe infection. Local adenosine levels are tightly regulated by metabolism, suggesting that effects via low affinity A_{2B} and A_3 receptors may not be of long duration. Adenosine acts through A_1 and A_{2A} receptors both in areas of severe inflammation with high local adenosine levels and areas with less severe inflammation and low adenosine levels. Acting through A_2 receptors, adenosine modulates the local immune response by decreasing neutrophil adhesion and activity (Bouma et al., 1997; Bullough et al., 1995; Revan et al., 1996; Sitaraman et al., 2001; Sullivan et al., 1999; Sullivan et al., 1990; Walker et al., 1997), decreasing local cytokine production (Hasko et al., 2000; Hasko et al., 1996; Le Moine et al., 1996; Link et al., 2000; Mayne et al., 2001; Ohta and Sitkovsky, 2001; Sajjadi et al., 1996; Sitkovsky, 2003; Xaus et al., 1999), and increasing maturation, activity, and MHCI-dependent antigen presentation of dendritic cells (Panther et al., 2002; Panther et al., 2001). It is interesting that A_2 receptors promote activation of CTL by MHCI-dependent antigen presentation, while decreasing CTL activity by A_3 receptors. These seemingly opposing actions suggest that adenosine may have different effects on CTL responses depending on the severity of the infection. Low adenosine levels favour control of innate immune responses through neutrophils and possible promotion of CTL responses through dendritic cells whereas high adenosine levels favour control of adaptive immune responses through T lymphocytes. It may be that higher,

more sustained adenosine levels are associated with degrees of infection that activate adaptive immune responses, necessitating a local modulator of that response. Lower adenosine levels may be associated with levels of damage or infection that require only innate immunity, necessitating a local modulator of neutrophil and monocyte function. Nevertheless, what is clear is that adenosine has complex and tightly controlled effects on immune cells and likely plays an important role in modulating whatever type of immune response is required. Our proposed model of control and modulation of immune responses by adenosine is summarized in Figure 2.

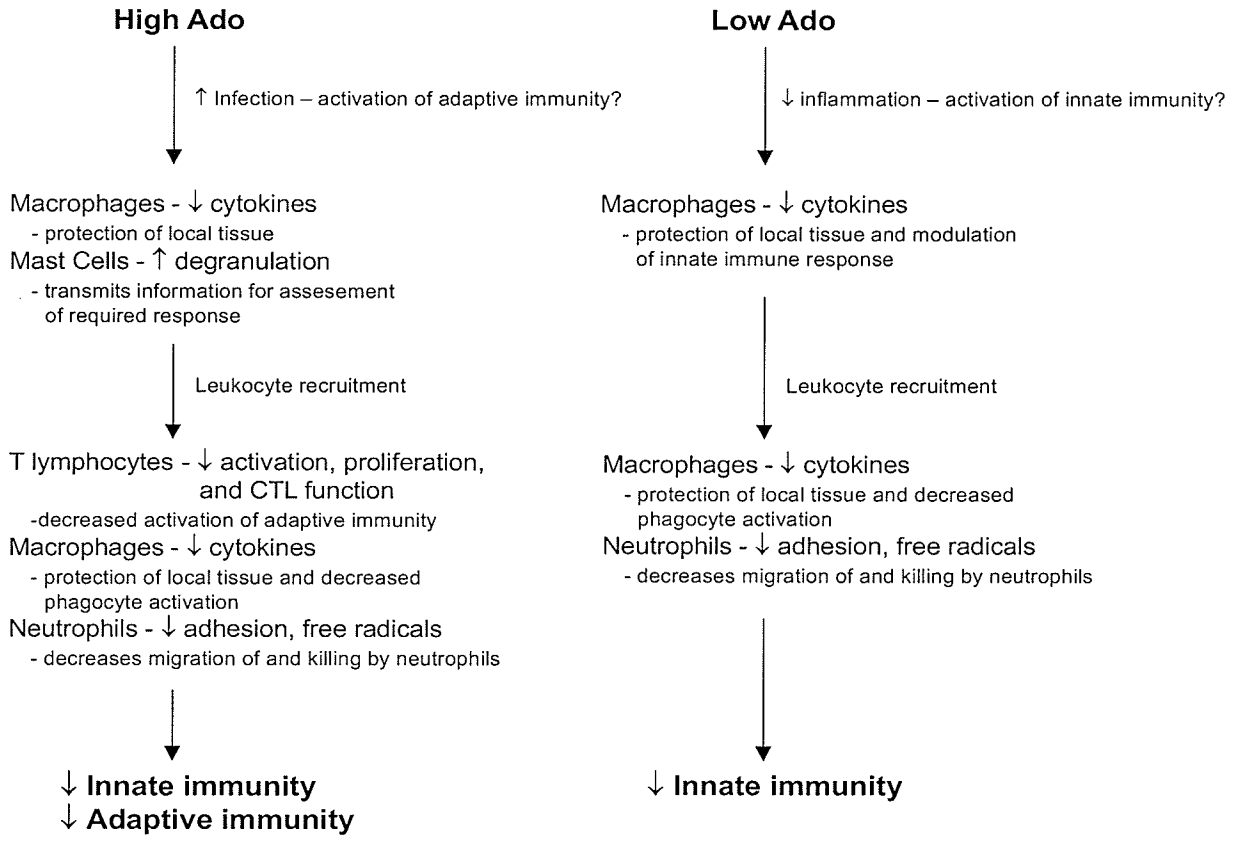

Figure 2: Proposed Model for Regulation of Immune Responses by Adenosine. Levels of high adenosine activate all 4 adenosine receptors, particularly low affinity A_{2B} and A_3 receptors. Levels of low adenosine activate high affinity A_1 and A_{2A} receptors.

Our studies on adenosine A_{2A} receptors contribute to this model by outlining signaling mechanisms involved in regulating cytokine production by macrophages. In our proposed model of adenosine-mediated immune response modulation, macrophages are activated initially, alongside mast cells, because they are tissue resident immune cells and respond by secreting cytokines that further activate macrophages and contribute to activation of leukocytes once recruited to inflammatory sites. Macrophages are activated during events that trigger both high and low adenosine levels, and adenosine controls cytokine production during both scenarios.

We propose that adenosine regulates TNF-α production by macrophages through A_{2A} receptors when adenosine levels are increased and when macrophages are activated during an initial inflammatory event. Control of TNF-α occurs post-transcriptionally by decreased stability and half-life of mRNA via a mechanism that may not be specific for TNF-α and may also regulate production of other transiently expressed proteins by macrophages during an inflammatory event. TNF-α regulation by A_{2A} receptors is maintained for up to 8-16 hours, suggesting that cytokine production is controlled by these receptors during initiation of both innate and adaptive immune responses. Regulation of TNF-α by A_{2A} receptors may contribute to tissue protection upon initiation of inflammation until the type of required immune response is determined because high levels of TNF-α can be toxic to healthy tissue. In addition, A_{2A} receptor-mediated control of TNF-α may help control macrophage, neutrophil, and T lymphocyte activation

and function because TNF-α contributes to the activation of these cells (Tracey, 2002). During events associated with high adenosine levels, mast cell degranulation causes recruitment of peripheral neutrophils, monocytes, and lymphocytes to the affected tissue (Nathan, 2002). Adenosine, via A_{2A} receptors, may modulate the extent of this response by decreasing adhesion of monocytes and subsequent migration into the tissue. Cytokines like TNF-α promote migration of cells by increasing expression of integrins on microglia and endothelial cells (Gao et al., 2002; Milner and Campbell, 2003) and by increasing endothelial cell adhesion (Javaid et al., 2003). Adenosine A_{2A} receptors may modulate cell adhesion and migration by decreasing integrin expression on monocytes, as our microarray data suggest. In addition, local control of pro-inflammatory cytokine levels by adenosine may modulate peripheral cell recruitment and migration by decreasing adhesion molecule expression on endothelial cells, although this potential functional effect of adenosine-mediated cytokine inhibition has not been studied.

Our data suggest that A_{2A} receptor activation controls TNF-α mRNA stability by decreasing activity of the p38 signaling pathway. p38 normally controls mRNA stability through ARE regions contained in the 3' UTR between the stop codon and the poly A tail. Proteins whose mRNAs contain AREs are generally transiently expressed, such as those expressed in response to stress and inflammation. Expression of these proteins is efficiently regulated by proteins that interact with ARE regions and control mRNA stability. By modulating the

activity of this signaling pathway, adenosine A_{2A} receptors may not only affect TNF-α production but production of other transiently expressed proteins associated with stress and immune responses. As such, adenosine may utilize this pathway to selectively target these types of proteins without affecting global protein expression. This specificity would likely be more difficult if A_{2A} receptor actions were pre-transcriptional or if they involved affects on general protein translational complexes.

Our studies have contributed both to our understanding of the role adenosine may play in regulating inflammatory and immune responses as well as our understanding of signaling mechanisms mediating the effects of adenosine A_{2A} receptors. In order to grasp the complex role adenosine plays in biological functions like immune responses, it is important to elucidate the roles of specific adenosine receptors and these studies have been difficult in vivo because of the relative paucity of animal models. In vitro studies with adenosine receptor agonists have helped identify some receptor specific actions of adenosine on immune cells but we must interpret these with caution, particularly for A_{2B} and A_3 receptors, because tissue distribution and functional expression of these receptors is not well known. Although mRNA for A_3 receptors has been found in most tissues, only eosinophils have been shown to express A_3 receptor protein by radioligand binding studies (Klinger et al., 2002). In addition, there is controversy as to whether mast cell degranulation by adenosine is mediated by A_{2B} or A_3 receptors and identification of A_{2B} receptor functions are exceedingly

difficult because no receptor selective agonists exist (Klinger et al., 2002). As a result, we are limited to in vitro studies like those we have conducted to identify important functions of the adenosine system as they relate to immune regulation. Our data provide important contributions to the understanding of A_{2A} receptor regulation of macrophage function and to how these actions may regulate downstream development of immune responses. However, further studies focused particularly on the role of low affinity adenosine receptors in immune system modulation are essential to further develop models similar to the one we have proposed here for immune response regulation by adenosine.

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Appendix

A 1.1 Cultured U937 Cells

Human pro-monocytic U937 cells (ATCC CRL 1593.2; batch F12641) were obtained from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 (pH 7.2) media supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 1% antibiotic/antimycotic (Chen et al., 1997). Cells were maintained at 37°C in a humidified growth chamber supplemented with 5% CO_2.

A 1.2 Cultured THP-1 Cells

Human THP-1 cells, a monocytic cell line, were cultured in RPMI 1640 media supplemented with 10% heat-inactivated FBS and 1% antibiotic/antimycotic solution. Cells were maintained at 37°C in a humidified growth chamber supplemented with 5% CO_2.

A 1.3 Primary Macrophage Cultures

Peripheral blood was isolated from healthy volunteers or from multiple sclerosis patients and was mixed 1:1 with fresh RPMI 1640 media prepared without serum. To separate erythrocytes, leukocytes, and serum, 25 ml of the blood/media mixture was loaded onto 10 ml Histopaque 1077 in 50 ml centrifuge tubes and centrifuged at 2000 rpm for 20 minutes. The layer of leukocytes lying above the histopaque cushion was removed, washed with fresh RPMI 1640, and centrifuged at 1500 rpm for 10 minutes. Leukocyte pellets were resuspended in 5 ml RPMI 1640, washed in 50 ml RPMI 1640, and centrifuged at 1000-1200 rpm for 15 minutes. At this speed, monocytes pelleted and platelets stayed in solution. Monocyte pellets were resuspended in RPMI 1640 supplemented with

15% FBS and 1% antibiotic/antimycotic. Monocytes were plated overnight in 175 cm^2, vent-capped, polystyrene culture flasks. After 24 hours, monocytes and macrophages adhered to culture flasks and were subsequently rinsed and scraped with RPMI 1640 supplemented with 10% FBS and 1% antibiotic/antimycotic. Monocytes and macrophages were plated in 96 and 6 well culture plates and allowed to differentiate for 7 days. Every two days, half of the monocyte and macrophage culture media was replaced with fresh RPMI 1640 supplemented with 10% FBS and 1% antibiotic/antimycotic. During differentiation of monocytes to macrophages in culture, cells take on an elongated phenotype with branched extensions and rounded morphology. Differentiation using this method increased expression of CD14, MHCII, and CD68 (MacKenzie et al., 2002).

A 1.4 ELISA

Purified anti-human TNF-α primary antibody (BD PharMingen cat# 551220) was prepared in 0.05 M carbonate-bicarbonate buffer (0.8 g/500ml Na_2CO_3, 1.47 g/500ml $NaHCO_3$, pH 9.6) to a final concentration of 2 μg/ml. Corning EIA/RIA 96 well plates were coated overnight at 4°C with 50 μl/well of 2 μg/ml primary antibody. Following overnight antibody coating, ELISA plates were washed 3 times with PBS-Tween (100 nM PBS pH 7.4, 0.05% Tween 20). Plates were blocked for 1 hr with 100 μl/well of 1% BSA in PBS. Plates were then washed 3 times with PBS-Tween and loaded with 50 μ/well of TNF-α standards and experiment supernatants in triplicate overnight at 4°C. TNF-α standards were

prepared in PBS from aliquots stored at –80°C (500ng/μl in 1% BSA) at concentrations of 5000, 2500, 1250, 625, 312, 156, 78, and 39 pg/ml. Following overnight incubation, ELISA plates were washed 3 times with PBS-Tween. Biotin mouse anti-human TNF-α (BD PharMingen cat#18642D) was prepared at a final concentration of 0.5 μg/ml in dilution buffer (0.5% BSA, 0.05% Tween in PBS) and 50 μl/well was added to ELISA plates for 30 minutes. After 30 minutes, plates were washed 3 times in PBS-Tween and incubated for 30 minutes with 50 μl/well of streptavidin-AKT (BD PharMingen cat# 554065) prepared as a 1:1000 dilution in dilution buffer. After 30 minutes, plates were washed 3 times in PBS-Tween and developed for 20 minutes with 50 μl/well of Sigma 104 Phosphatase substrate tables (p-Nitrophenyl phosphate) prepared at 1mg/ml in substrate buffer (100 mg/L $MgCl_2 \cdot 6H_20$, 10% diethanolamine, pH 9.8). After developing for approximately 20 minutes, absorbance was read on a spectrophotometer at 405-620 nm. TNF-α levels in experiment supernatants were calculated from TNF-α standard curves.

A 1.5 RT-PCR

Total RNA was prepared from cell cultures using a GenElute Mammalian Total RNA Purification kit (Sigma-Aldrich, Oakville, ON). Cell culture pellets were lysed in 250 μl of lysis solution (1% 2-mercaptoethanol) and centrifuged at 15,000 rpm for 2 minutes in filtration columns to remove cellular debris and to shear DNA. Lysates were mixed with an equal volume of 70% ethanol to precipitate nucleic acids. Lysate-ethanol mixes were centrifuged for 15 seconds

at 15,000 rpm in binding columns to bind precipitated nucleic acid to filter. Binding column filters were washed once using wash solution 1 and twice using wash solution 2. After each wash, columns were centrifuged for 15 seconds at 15,000 rpm. During the final wash, columns were centrifuged at 15,000 rpm for 2 minutes to dry the binding columns. Binding columns were transferred to fresh collection tubes and nucleic acid was eluted with 50 μl of elution solution (RNase-free H_20). Total RNA was stored at –80°C or used immediately for cDNA synthesis. To remove DNA from RNA samples for cDNA synthesis, 8 μl of total RNA was mixed with 1 μl of 10X reaction buffer (Sigma DNase kit) and 1 μl of Amplification Grade DNase I in 200 μl PCR tubes for 15 minutes at room temperature. After 15 minutes, 1 μl of stop solution was added to each tube to bind calcium and magnesium and inactivate DNase I. Tubes were heated at 70°C for 10 minutes to denature DNase I and RNA. RNA was then reverse transcribed into cDNA using a First Strand cDNA synthesis kit (MBI Fermentas, Burlington, ON). Random hexamer primer was added to each sample while still hot (1 μl of 0.1 μg/μl) and chilled on ice. Each sample was mixed with 5X reaction buffer (4 μl), ribonuclease inhibitor (1 μl of 20 U/μl) and 10 mM dNTP mix (2 μl) and incubated at 25°C for 5 minutes. M-MuLV reverse transcriptase (2 μl per tube of 20U/μl) was added and mixtures were incubated at 25°C for 10 minutes followed by 37°C for 60 minutes. Reactions were terminated by heating tubes for 10 minutes at 70°C. cDNA was stored at –20°C or used directly for PCR. cDNA was PCR amplified using a Taq PCR Master Mix kit (Qiagen). PCR master mix was prepared as per manufacturers directions (Per reaction: 12.5 μl

of Taq PCR master mix, 2.5 μl each of 3' and 5' primers (1 μM final concentration), and 4.5 μl H_2O). 22 μl of master mix was added to 3 μl of cDNA product using either TNF-α (5' GACCTCTCTCTAATCAGCCC, 3' CAAAGTAGACCTGCCCAGAC), GAPDH (5' ACCACCATGGAGAAGGCTGG, 3' CTCAGTGTAGCCCAGGATGC), β-actin (5' TGGTGGGCATGGGTCAGAAG, 3' GTCCCGGCCAGCCAGGTCCAG) or cyclophillin (5' GCTGCGTTCATTCCTTTTG, 3' CTCCTGGGTCTCTGCTTTG) specific primers. TNF-α, β-actin, and GAPDH PCR reactions were run for 25 cycles at 95°C denaturation for 30 seconds, 53°C annealing for 30 seconds, and 72°C extension for 30 seconds. Cyclophillin PCR reactions were run for 40 cycles with denaturation for 15 sec at 95°C, annealing for 15 sec at 60°C, and extension for 15 sec at 72°C. RT-PCR is semi-quantitative and to ensure that 25 cycles produced a quantity of PCR product within the linear range of the amplification curve for the primers used, cDNA prepared from PMA/PHA stimulated U937 cells was amplified to create a curve of TNF-α PCR product produced from increasing numbers of cycles (5, 10, 15, 18, 20, 22, 24, 26, 28, 30, 32, 35, 40 see Figure 2.7). PCR products were separated by agarose gel electrophoresis (1.5% agarose, 60 ng/ml ethidium bromide in TBE) at 100V for 35 minutes and imaged under UV light using the BioRad Gel Documentation System.

A 1.6 Western Blots

Whole cell lysates were prepared from cell pellets using 2X gel loading buffer (100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 20% glycerol and 0.2%

bromophenol blue). Twenty μl of whole cell lysates was loaded on SDS-PAGE gels in addition to 10 μl of BioRad broad range pre-stained protein standard (205 kD – 6kD). For western blots with phospho-specific antibodies, cell pellets were lysed for 1 hour on ice in phosphorylation lysis buffer. Protein extracts were centrifuged at 18 000 x g for 10 minutes at 4°C. Supernatants were collected and added to equal volume 2X gel loading buffer. Twenty μl of this extract was loaded onto SDS-PAGE gels. Depending on the size of the protein of interest, gels were typically 8-12% acrylamide. Composition of resolving gels were 375 mM Tris pH 8.8, 0.1% SDS, acrylamide/bisacrylamide, 0.05% ammonium persulfate, 0.05% Temed. Composition of stacking gels were 125 mM Tris-HCl pH 6.8, 0.1% SDS, 4% acrylamide/bisacrylamide, 0.1% ammonium persulfate, 1% Temed. Gels were run in ice-cold running buffer (5 mM Tris, 50 mM glycine, 0.02% SDS pH 8.3) at 50V for approximately 15 minutes followed by 150V for approximately 1 hour. Gels and nitrocellulose membranes were incubated in cold transfer buffer (39 mM glycine, 48 mM Tris, 0.037% SDS, 20% methanol pH 8.3) for 20 minutes prior to transferring to allow transfer buffer to equilibrate in gels and nitrocellulose membranes. Gels were transferred onto nitrocellulose membranes using the BioRad Trans-Blot SD Semi-Dry Transfer Cell for 30 min at 15V. Following the transfer, nitrocellulose membranes were placed in blocking buffer (1% Western Blocking Reagent from Roche Diagnostics cat# 1921673 in TBS) overnight at 4°C. Primary antibodies were prepared in 0.5% western blocking reagent in TBS and incubated with nitrocellulose membranes at room temperature for 4 hours or at 4°C overnight. Membranes were washed 3 times

with TBS-Tween (1% Tween), rinsed once with TBS and incubated for 1 hour with horseradish peroxidase conjugated secondary antibody. All secondary antibodies were IgG H+L antibodies obtained from Jackson ImmunoResearch Laboratories and were prepared in 0.5% western blocking reagent in TBS at final concentrations recommended by the manufacturer (150 ng/ml for secondary anti-goat and 200 ng/ml for secondary anti-rabbit and anti-mouse). Bands were visualized using ChemiGlow West Substrate (Alpha Innotech, San Leanardo, CA). Membranes were incubated for 5 minutes in working solution prepared with equal volumes of ChemiGlow West luminal/enhancer solution and stable/peroxide solution. After 5 minutes, membranes were removed, placed in plastic sheets, and protein bands were imaged and quantified using a BioRad FluorS Max imaging system.

A 1.7 Electrophoretic Mobility Shift Assay

Cell culture pellets were resuspended in 20 μl of Totex buffer (20 mM HEPES pH 7.9, 350 mM NaCl, 20% glycerol, 1% NF-40, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, aprotinin, 0.5 M DTT, 0.1% PMSF) and incubated on ice for 30 minutes. The cell lysates were centrifuged for 10 minutes at 15,000 rpm at 4°C and supernatants were removed and stored at –80°C. [γ-^{32}P]ATP end-labelled transcription factor consensus oligonucleotides were prepared as per manufacturer's instructions (Promega). Consensus oligonucleotide (1.75 pmol) was incubated at 37°C for 30 minutes with 10x buffer kinase, [γ-^{32}P]ATP, ddH_20, and T4 kinase. Free [γ-^{32}P]ATP was separated from [γ-^{32}P]ATP end-labelled

oligonucleotides using a sephadex column. To incubate experimental protein samples with [γ-^{32}P]ATP end-labelled oligonucleotide, 20 μg of protein was added to a 1.7 ml microcentrifuge tube to a final volume of 11.4 μl with 10.6 μl EMSA mix (20 µg BSA, 1 µg dIdC, 2X Buffer F, 0.5X Buffer D, 0.7 µl [γ-^{32}P]ATP end-labelled oligonucleotide per sample) and incubated at room temperature for 15-20 minutes. Protein-EMSA mix was run on a 7% acrylamide/bisacrylamide gel at 200 V for 1-2 hours. Gels were dried using a BioRad Gel Dryer for 2 hours at 80°C. Dried gels were placed with Kodak X-Omat film in cassettes at –80°C for 2-3 days prior to developing. Developed films were scanned and pixel density of bands determined with Scion Image (NIH software).

A 1.8 Serine/Threonine Protein Phosphatase Assays

Serine/threonine protein phosphatase activity was determined using a commercially available enzyme assay kit (New England Biolabs). Briefly, myelin basic protein (MyBP) was labeled on serine and threonine residues with [γ-^{33}P] ATP by protein kinase A. Experimental protein extracts containing active protein phosphatases were incubated with ^{33}P labeled MyBP. Protein phosphatase activity was determined by measuring ^{33}P released from MyBP during incubation with experimental protein extracts, indicating degree of dephosphorylation of labeled MyBP.

To label MyBP, the following were mixed together in a 1.5 ml eppendorf tube and incubated overnight at 30°C:

PKA/MyBP mix	40 μl
10X PKA buffer	20 μl
water	115 μl
ATP (10mM)	20 μl
^{33}P-ATP	5 μl

A 2 μl sample of the mixture was diluted 1:100 in water and radioactivity in 2 x 10μl aliquots was determined by a scintillation counter to determine the specific activity of ATP. Specific activity of ATP was determined as follows:

ATP specific activity (cpm/pmol) = aliquot cpm x 1/10 x 100 x 200 x 1/200000

-10 is the aliquot volume (μl)
-100 is the dilution factor
-200 is the volume of the reaction (μl)
-200,000 is the amount of pmol ATP in the 200 ul reaction

The reaction was terminated by adding 1/9 total volume of cold 100% TCA. Samples were left on ice for 30 minutes. Radioactive ATP was removed by centrifugation at 12 000 x g for 10 min at 4°C, this step will remove most of the radioactive ATP. Supernatant was removed without touching the pellet and the pellet was washed three times with 1 ml of 20% TCA. Following the third wash, 0.5 ml of substrate solubilization buffer was added to solubilize the pellet, which dissolved in approximately 5-15 minutes. Once the pellet was solubilized, the sample was transferred to a dialysis bag, the sample tube was washed with an additional 0.5 ml of sample solubilization buffer and transferred into the dialysis

bag. The sample was dialyzed for 16 hours at 4°C against 2 L of dialysis buffer to remove residual radioactive ATP. Duplicate 5 μl aliquots were counted using a scintillation counter to determine radioactivity. The incorporated phosphate concentration (μM) was calculated using the original ATP specific activity.

$$\text{Phosphate Concentration } (\mu M) = \text{Aliquot cpm/Specific Activity} \times 1/5 \times 10^{6} \times 10^{-6}$$

-5 is the aliquot volume (μl)
-10^{6} is to convert the result for 1 liter rather than 1 μl
-10^{-6} is to convert the amount of phosphate from pmoles to umoles

The labeled MyBP was diluted in 1X protein phosphatase buffer to a concentration of 50 μM (5X concentration required for the enzyme assay) with respect to the incorporated ^{33}P.

Experiments were performed on U937 cells cultured to a density of 1 000 000 cells/ml in RPMI 1640 media supplemented with 10% FBS. Following experimental treatments, cells were collected on ice and pellets were washed once in ice cold PBS. After washing, cell pellets (approximately 10 X 10^{6} cells/pellet) were resuspended and homogenized in 50 μl homogenization buffer (see A 1.13). Whole cells, nuclei and large debris were removed by centrifugation for 10 minutes at 300 X g at 4°C. Supernatant was collected and centrifuged for 20 minutes at 18 000 X g at 4°C. Supernatant was collected (cytosolic fraction) and pellets (membrane fraction) were resuspended in 50 μl of

1X protein phosphatase buffer (NEB kit). Protein content in both fractions was determined by Bradford assay (BioRad Laboratories).

100 ng of protein from membrane or cytosol fractions was added to 1X protein phosphatase buffer (NEB kit) to a total of 40 μl. Eppendorf tubes containing protein and phosphatase buffer were pre-incubated at 30°C for 2-5 minutes prior to start of enzyme assay. ^{33}P labeled MyBP was added to each tube (10 μl) and incubated at 30°C for 10 minutes. Reactions were terminated by adding 200 μl of ice cold 20% TCA to each tube. Tubes were kept on ice for 5-10 minutes and centrifuged at 12 000 X g for 5 minutes. Supernatants containing released ^{33}P were collected (200 μl) and added to 2 ml scintillation fluid. Radioactivity was measured using a scintillation counter. Specific activity of serine/threonine protein phosphatases in experimental cell fractions was determined by:

Activity (nmol/min) = (sample cpm – blank cpm)/(total cpm – blank cpm) x 0.5/10 x 20 x 250/200

-0.5 is the nmoles of incorporated phosphates in the assay (10 μl of labeled MyBP in 50 μl assay volume)
-10 is the duration of the assay in minutes
-20 converts the volume units to ml from the 50 μl reaction
-250/200 corrects of the fraction of TCA supernatant counted

Protein Calculation (mg) = extract protein concentration (μg/μl) x X / 50

-X is the volume of protein extract added to the assay (μl)
-50 is the volume of the final assay reaction (μl)

Specific Activity (nmol/min/mg) = Activity/Protein Calculation

A 1.9 Immuno Microarrays

Total RNA was extracted from cell pellets using the same protocol as for RT-PCR (see A 1.5). DNA microarrays were performed as described previously (Mayne et al., 2001). Nylon arrays were blocked using a cocktail containing Microhybridization buffer, Cot1 DNA and polyA. Dry arrays were pre-wet in 2X SSC to keep DNA attached to nylon. Arrays were then placed in 50 ml centrifuge tubes, ensuring there were no bubbles between array and plastic wall of tube, with 4ml of blocking buffer. Arrays were rotated in a hybridization oven at 42°C for 4 hours. To synthesize ^{33}P-dCTP labeled cDNA from RNA samples, 15 μl of total RNA was denatured at 70°C for 5 minutes. After adding oligo-dT the mix was then heated at 70°C for 5 minutes and placed on ice. To each sample 17 μl cocktail (denatured RNA and oligo-dT), 5 μl ^{33}P-dCTP, and 2μl superscript was added on ice. Samples were incubated at 42°C for 35 minutes, spiked with 2 μl superscript and incubated at 42°C for an additional 35 minutes. Reactions were terminated by adding EDTA and NaOH and heating at 65°C for 30 minutes. To each sample, we added 25 μl of 1 M Tris-HCl pH 8.0 and separated free ^{33}P-dCTP from labeled cDNA using gel spin columns. Labelled cDNA was denatured at 95°C for 5 minutes and added to hybridization buffer in centrifuge tubes. Arrays hybridized at 42°C overnight and then exposed to phosphorimager screens. Images of the arrays were digitized using a Storm Phosphorimager.

A 1.10 Cell Adhesion Assays

Binding of THP-1 cells was determined using a fluorescence based cell adhesion assay. Cell cultures were loaded with the calcien-AM fluorophore, for 30 minutes

prior to determining cell adhesion. Calein-AM is esterase cleaved and fluoresces in living cells. Cells were treated with adenosine receptor agonists for 30 minutes. Following the agonist incubation, levels of adhesion in unstimulated cultures were determined. For some experiments, cells were stimulated with ionomycin, PMA, or lipopolysaccharide for 15 minutes prior to determining cell adhesion. To detect cell adhesion, cell cultures were incubated on fibronectin-coated (10 μg/ml prepared in PBS overnight) 96 well black plates for 30 minutes to allow cells to adhere. Non-adherent cells were washed off with PBS, and fluorescence was measured at excitation 485 nm and emission 530 nm on an automated plate reader. Data was expressed as a ratio of specific fibronectin-mediated binding over non-specific binding to uncoated black plates. Determining fibronectin-specific binding reflects adhesion mediated by α4β1-, α4β7-, α5β1-, α8β1-, α3β1-, αVβ1-, αVβ3-, αVβ6-, αVβ8-, and αIIbβ3-integrins (Milner and Campbell, 2002).

A 1.11 Protein Measurements

Protein content in cell lysates was determined using the BioRad protein assay (BioRad Laboratories). This assay is based on the Bradford procedure. Aliquots of cell lysates (1-10 μl) were measured along with 10 μl aliquots of bovine serum albumin protein standards (0.5, 1, 2, 3, 4, 5 mg/ml) and combined with 200 μl of BioRad protein assay reagent. The colour change of the reaction results from Coomassie Brilliant Blue dye which binds to basic and aromatic amino acid residues. Absorbance was measured at 595 nm and protein concentrations of samples were extrapolated from bovine serum albumin protein standard curves.

A 1.12 Statistics

For all experiments other than DNA microarrays, statistical significance was determined using ANOVA followed by Tukey's multiple comparison test to determine differences between all groups within each experiment. Data are expressed graphically as mean ± standard error and are presented as % PMA/PHA with 100% corresponding to 467 ± 104 pg TNF-α in 200 μl cell supernatant.

For DNA microarrays, Z scores were calculated for each gene on the array using the following equation:

$$\text{Z score} = [\log_{10}(\text{individual gene}) - \log_{10}(\text{average gene expression for array})]/\log_{10}(\text{standard deviation for array})$$

In Tables 4.1, 4.2, and 4.3 data are presented as Z ratios which were calculated as follows:

$$Z = (X_1 - X_2)/\sqrt{\ [(SD(X_1)^2)/N(X_1) + (SD(X_2)^2/N(X_2)]}$$

In the above equation, X denotes the individual groups being compared, SD(X) denotes the standard deviation and N(X) denotes the number of values for each group being compared. P values were calculated using two-tailed T tests.

A 1.13 Buffers

PBS
137 mM NaCl
2.7 mM KCl
10 mM Na_2HPO_4
2 mM KH_2PO_4
pH 7.4

TBS
137 mM NaCl
2.7 mM KCl
24.8 mM Tris Base
pH 7.4

Totex Buffer
20 mM HEPES pH 7.9
350 mM NaCl
20% glycerol
1% Igepal
1 mM $MgCl_2$
0.5 mM EDTA
0.1 mM EGTA
Aprotinin
DTT
PMSF

5X Buffer F
20% Ficoll 400
100 mM HEPES pH 7.9
300 mM KCl
10 mM DTT
0.1 mM PMSF

Buffer D
20 mM HEPES pH 7.9
20% glycerol
100 mM KCl
0.5 mM EDTA
0.25% Igepal
2 mM DTT
0.1 mM PMSF

PSP Homogenization Buffer
1 mM EDTA
2.5 μg/ml chymostatin
1.0 μg/ml pepstatin
5.0 μg/ml antipain
2.5 μg/ml N^a-p-topsyl-L-lysine chloromethyl ketone (TLCK)
1. 0 μg/ml leupeptin
0.025 trypsin inhibitory units/ml aprotinin

PSP Dialysis Buffer
25 mM Tris-HCl pH 7.5
0.1 mM Na_2EDTA
2 mM DTT
0.01% Brij 35

PSP 1X PKA Buffer
50 mM Tris-HCl pH 7.5
10 mM $MgCl_2$
1 mM EGTA
2 mM DTT
0.01% Brij 35

Substrate Solubilization Buffer
50 mM Tris-HCl pH 8.5
0.1 mM Na_2 EDTA
2 mM DTT
0.01% Brij 35

PSP 1X Protein Phosphatase Buffer
50 mM Tris-HCl pH 7.0
0.1 mM Na_2EDTA
5 mM DTT
0.01% Brij 35

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