MODULATION OF THE INFLAMMATORY RESPONSE
BY TRIPTOLIDE AND MAP KINASE PHOSPHATASE-1

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
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By

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ABSTRACT

The primary function of the immune system is to prevent disease by detecting and eliminating pathogenic organisms or host’s abnormal cells as a result of infection or mutation while avoiding self-destruction. A delicate balance must be achieved between the timely and appropriate activation of the immune system and subsequent deactivation when pathogens abnormal cells are cleared. An adequate production of pro-inflammatory cytokines plays a crucial role in effective host immune defense, and abnormal pro-inflammatory cytokine production can cause various illnesses. The immune system utilizes a variety of mechanisms to achieve adequate immune responses including balanced cytokine production to combat the diverse and ever changing microorganisms. Feedback control through the induction of either intracellular or secreted regulators is a major mechanism employed by the immune system to fine-tune the host responses. Modulation of the immune response pathways by ingredients of certain plants may account for the therapeutic benefits observed in alternative medicines. This thesis will focus on the regulation of the inflammatory response by two completely different immune modulators: MAP kinase Phosphatase-1, an endogenous protein phosphatase, and triptolide, a small molecule isolated from an anti-rheumatic herb in Chinese medicine, T. wilfordii Hook F. In Chapter 1, we will briefly review our current
understanding of the immune response to pathogenic organisms, and introduce the basic signaling pathways as well as the key players involved, including MKP-1. We will also briefly summarize the properties of triptolide, and our understanding of its anti-inflammatory mechanisms. Chapter 2 will include a detailed description of our studies on the therapeutic mechanism of triptolide as an anti-inflammatory agent. In Chapter 3, we will switch to MKP-1, and address the basic mechanism via which MKP-1 regulates the innate immune responses. This chapter will focus on the effects of $Mkp-1$ knockout on cytokine mRNA stability. In Chapter 4, we will present our work on the role of MKP-1 in the pathogenesis of inflammatory bowel disease, using an IL-10 knockout mouse model. Finally, we will summarize our findings and propose a general model illustrating the modes of action of MKP-1 and triptolide in the modulation of inflammatory responses.
Dedicated to my mother and father, without their prayers and support this degree would not have been possible.
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FIELDS OF STUDY

Major Field: Integrated Biomedical Science
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<td>AEC</td>
<td>3-Amino-9-Ethylcarbazole</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activating protein-1</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cells</td>
</tr>
<tr>
<td>ARE</td>
<td>AU-rich element</td>
</tr>
<tr>
<td>ATF</td>
<td>activating transcription factor</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BIC</td>
<td>B-cell integration cluster</td>
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<tr>
<td>CBP</td>
<td>cAMP response element (CREB) -binding protein-binding protein</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>CD4</td>
<td>cluster of differentiation 4</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<td>COX</td>
<td>cyclooxgenase</td>
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<tr>
<td>CpG</td>
<td>cytosine phosphodiester guanine</td>
</tr>
<tr>
<td>CSF</td>
<td>colony stimulating factor</td>
</tr>
<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole</td>
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<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>DUSP</td>
<td>dual specificity protein phosphatase</td>
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<tr>
<td>EIM</td>
<td>extra-intestinal manifestation</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>EMSA</td>
<td>electromobility gel-shift assay</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
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<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>Hprt</td>
<td>hypoxanthine-guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>HSF</td>
<td>heat shock factor</td>
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<tr>
<td>Hsp</td>
<td>heat shock protein</td>
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<tr>
<td>IkB</td>
<td>inhibitor of κB</td>
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<tr>
<td>IBD</td>
<td>inflammatory bowel disease</td>
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<tr>
<td>IC50</td>
<td>inhibitory concentration</td>
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<td>ICAM</td>
<td>inhibitors of cell adhesion molecules</td>
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<td>IFN</td>
<td>Interferon</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>IKK</td>
<td>inhibitor of κB kinase</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin (i.e. IL-10 = interleukin-10)</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>IL-1 receptor antagonist</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IRAK</td>
<td>IL-1R-associated kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>MAPK</td>
<td>Mitogen-Activated Protein Kinase</td>
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<tr>
<td>MAPKKK</td>
<td>MAPK Kinase Kinase</td>
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<tr>
<td>MD-2</td>
<td>myeloid differentiation protein 2</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>miR</td>
<td>micro-RNA</td>
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<tr>
<td>MK-2</td>
<td>MAP kinase-activated protein kinase-2</td>
</tr>
<tr>
<td>MKK</td>
<td>MAPK Kinase</td>
</tr>
<tr>
<td>MKP</td>
<td>MAP kinase phosphatase</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation factor 88</td>
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<tr>
<td>NEMO</td>
<td>NF-κB essential modulator</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa B</td>
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<tr>
<td>NF-AT</td>
<td>nuclear factor of activated T-cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOD</td>
<td>nucleotide binding oligomerization domain</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PAMPs</td>
<td>pathogen-associated molecular patterns</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PepG</td>
<td>peptidoglycan</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative real-time PCR</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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</table>
S.E.                standard error
siRNA              small interfering RNA
SOCS               suppressor of cytokine signaling
SPF                specific pathogen-free
STAT               signal transducer and activator of transcription
TA                 transactivation
TBP                TATA-binding protein
TCR                T cell receptor
TD-101             acetylated triptolide
TetR               tet repressor
TFII               transcription factor for RNA polymerase II
TGF-β              transforming growth factor-beta
T<sub>h</sub>       T helper
TJ                 tight junction
TLR                Toll-like Receptor
TNF-α              tumor necrosis factor-alpha
TRAF-6             TNF receptor associated factor 6
TRAM               TRIF-related adaptor molecule
TRIF               Toll/IL-1 receptor domain-containing adapter inducing interferon-β
TTP                tristetraprolin
TwHF               *Tripterygium wilfordii Hook F.*
UC                 ulcerative colitis
UTR                untranslated region
<table>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>VEGF</td>
<td>vascular epithelial growth factor</td>
</tr>
<tr>
<td>VP16</td>
<td>herpes simplex virus protein 16</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zonula Occludens</td>
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</table>
CHAPTER 1

INTRODUCTION

1.1 The Immune System

The immune system is a collection of specialized cells and biological processes within an organism which interact in an elaborate and dynamic network and protect against disease by identifying and killing pathogens (1). The immune system can detect a wide variety of pathogenic agents, from viruses to parasitic worms, and needs to distinguish them from the organism's own healthy cells and tissues in order to function properly. The primary function of the immune system is to prevent diseases by detecting and eliminating microbial pathogens while preserving healthy tissues (2). This is achieved by a delicate balance between a timely and appropriate activation of the immune system and subsequent deactivation when the pathogens are cleared. Pathogens can evolve rapidly, producing adaptations that evade the immune system and allow these pathogens to successfully infect their hosts. To overcome this challenge, multiple mechanisms evolved allowing the immune system to recognize and neutralize pathogens. These mechanisms include antimicrobial peptides called defensins, phagocytosis, and the complement system. The immune system adapts over time to recognize specific
pathogens more efficiently. This adaptation process is referred to as "adaptive or "acquired immunity" and creates immunological memory. Immunological memory created from a primary response to a specific pathogen, provides an enhanced response to secondary encounters with that same, specific pathogen. This process of acquired immunity is the basis of vaccination. The mammalian immune system is broadly divided into two major components: the innate immunity and the adaptive immunity (2).

The Innate Immune System: The innate immune system detects a wide variety of non-self organisms through a limited set of germ line-encoded receptors, and serves as a first line of defense against pathogenic organisms (3). Innate immune defenses are non-specific and do not confer long-lasting immunity against a pathogen (1). Innate host defense can be classified into traditional and non-traditional defenses.

The traditional cellular innate host defense is comprised of a number of cell types, including dendritic cells, macrophages, monocytes, natural killer (NK) cells, neutrophils, eosinophils, and mast cells. Neutrophils and macrophages are phagocytic cells that engulf and destroy microorganisms. Dendritic cells and macrophages are professional antigen-presenting cells. Mast cells store histamine and play an important role in allergic reactions (1). NK cells are cytotoxic cells that are important in immunity to viruses and intracellular pathogens (1). Innate immune effector cells recognize a limited range of conserved molecules common to many pathogenic organisms, which are often essential for the survival of these pathogenic organisms. The innate immune system can provide immediate nonspecific defense and prevent rapidly dividing pathogens from overwhelming the host.
In addition to cellular innate immune defense, innate immunity also includes other defense mechanisms utilized by the host in a pathogen-independent manner. Multicellular organisms often use many barriers to protect themselves from microbial infection, including physical, chemical and microbiological barriers. The surface of the body is protected by a continuous layer of epithelial cells which forms a barrier between the body's internal environment and the external surroundings (1). These epithelial cells comprise the skin, the mucosal epithelial linings of the respiratory, gastrointestinal (GI), and urogenital tracts. Mucosal surfaces are constantly covered with a layer of mucus that contains glycoproteins and enzymes. Mucus can trap microorganisms, thus serves as an additional barrier to protect epithelial cells from damage and limit infection. Lysozyme in saliva and tears degrades bacterial cell walls (1). In the respiratory tract, the mucus is continuously removed through the action of epithelial cells bearing beating cilia. The acidic environments of the stomach and skin hinder microorganism invasion (1).

In addition to the barrier defense mechanism, epithelial cells of the epidermis, respiratory tract, and gastrointestinal tract can eliminate microorganisms on the surface by secreting antimicrobial peptides known as β-defensins. Defensins effectively destroy microorganisms on the epithelium by perturbing the membranes of bacteria, fungi, and enveloped viruses (1). Furthermore, most epithelia are coated with a flora of non-pathogenic microorganisms (microbiological barriers) that compete with pathogens for nutrients and for attachment sites on epithelial cells (1). Some commensal flora in the GI tract secrete anti-bacterial proteins which inhibit colonization by pathogens while others process digested food and synthesize vitamins (1). With such defenses, the skin and
mucosal surfaces provide well-maintained physical and chemical barriers that prevent pathogens from gaining access to the cells and tissues of the body.

Infections occur when these barriers are breached or when pathogens colonize the surfaces or the epithelial cells themselves (1). One of the first components of innate immunity to be activated is complement. The complement system is a biochemical cascade that attacks the surfaces of foreign cells. It contains over 20 different proteins which complement the antigen-binding function of antibodies in the defense against pathogens. Complement is the major humoral component of the innate immune response (4). Activation of the complement system initiates a series of enzymatic reactions in which the proteolytic cleavage and activation of successive complement components lead to the covalent binding of complement fragments to the pathogen surface. Phagocytes carry surface receptors that recognize these fragments; this recognition facilitates the uptake and destruction of complement-coated microbes by neutrophils and macrophages (1).

Microorganisms or toxins that successfully gain entry to an organism will encounter the cells and mechanisms of the innate immune system. The innate response is triggered when microbes are identified by pattern recognition receptors, which recognize components that are conserved among broad groups of microorganisms (5).

Toll-like Receptors and Signaling Pathways: Upon microbial infection, innate immune effector cells such as macrophages and dendritic cells localized in the affected tissues detect the pathogen-associated molecular patterns (PAMPs) through their specialized receptors including Toll-like receptors (TLR) and nucleotide binding oligomerization
domain-containing (NOD) proteins (6, 7). TLRs are a group of leucine-rich receptors evolutionally conserved from Drosophila to plants to mammals. To date, 10 members of the TLR family have been identified, each interacting with a specific class of ligands. Lipopolysaccharide (LPS), which is a component of Gram-negative bacteria, interacts primarily with TLR4. Peptidoglycan and lipoteichoic acid (components of Gram-positive bacteria) are recognized by TLR2, while flagellin interacts with TLR5. The CpG-rich motifs of bacterial DNA are detected by TLR9. Tri-acyl lipopeptides of mycobacteria are recognized by TLR1, whereas the di-acyl lipopeptides of mycoplasma interact with TLR6. Double-stranded RNA of viruses is recognized by TLR3. Recent studies have suggested that TLR7 is likely responsible for detecting single-stranded RNA. However, the ligands for TLR8 and TLR10 remain unclear. (8)

The detection of pathogen components by the pattern recognition receptors triggers a cascade of evolutionarily conserved signal transduction events mediated by adaptor proteins, including myeloid differentiation factor 88 (MyD88) and/or Toll/IL-1 receptor domain-containing adapter inducing interferon-β (TRIF), which in turn activate the NF-κB and mitogen-activated protein (MAP) kinase pathways (8). Ligand binding to TLRs leads to MyD88 recruitment, resulting in the formation of a signaling complex composed of the IL-1R-associated kinases (IRAK1 and IRAK4), plus the cytoplasmic adaptor TNF receptor associated factor (TRAF) 6 (9, 10). Phosphorylation of IRAK1 by IRAK4 leads to the activation of TRAF6 that in turn recruits the Ubc13/Uev1A E2 complex. The TRAF6/Ubc13/ Uev1A complex functions as an ubiquitin (E2 or E3) ligase which activates TAK1 in an ubiquitin-dependent manner (9, 11). TAK1 is necessary for activating inhibitor of κB (IκB) kinase, referred to as IKK, resulting in
phosphorylation and degradation of IκB, which releases and enables NF-κB to translocate to the nucleus.

The NF-κB family of transcription factors is composed of homodimers and heterodimers of five related proteins [NF-κB1 (p105/p50), NF-κB2 (p100/p52), c-Rel, RelA (p65), and RelB] (12). In unstimulated cells, they are held as inactive complexes in the cytoplasm by IκB proteins. TLR signaling via the TAK1, a MAP kinase kinase kinase (MAPKKK), leads to the activation of the IKK complex. The IKK complex comprises two catalytic (IKKα and IKKβ) and a regulatory (IKKγ, often referred to as NEMO) subunit (13-15). IKK-mediated phosphorylation of IκB targets it for ubiquitin-dependent proteasome-mediated degradation, thereby releasing NF-κB to be translocated to the nucleus where it regulates the transcription of genes with a NF-κB-binding site(s). Many cytokine genes and immune regulatory genes have the recognition and binding decameric sequences (κB elements) that can interact with NF-κB transcription factors.

In addition to regulating the NF-κB pathway, TAK1 also phosphorylates two members of the MAP Kinase Kinase family, MKK3 and M KK6, which in turn phosphorylate the stress-activated protein kinases, p38 and JNK, respectively (9, 16). Although this mode of MyD88-dependent NF-κB, JNK and p38 activation is utilized by the majority TLRs, TLR4 can also promote the activation of these signaling pathways by recruiting the alternate adaptor protein, TRIF (17). TRIF does not directly interact with this TLR; another adaptor TRAM (TRIF-related adaptor molecule), which is necessary for TRIF-dependent signaling downstream of TLR4 acts as a bridge between TRIF and TLR4 (9, 18). TLR3 signals almost exclusively through TRIF which activates NF-κB
and the IKK complex (9, 19). TAK1 activates both JNK and p38, whereas another MAPKKK, Tpl2 activates the ERK pathway downstream of most TLRs (20, 21).

**The Adaptive Immune System:** The adaptive immunity is a pathogen-specific host defense mechanism based on clonally expanded B- and T-lymphocytes generated first through random somatic recombination of genes encoding for the antigen receptors, and then matured through positive and negative selections. These pathogen-specific B- and T-lymphocytes not only serve as effector cells for pathogen eradication, but they also function as memory cells for rapid expansion upon re-encountering these pathogens. The adaptive immunity is highly specific and effective against evolving pathogens. If the host has never encountered a given pathogen, there will only be a few pathogen-specific lymphocytes in the host. Therefore, an initial effective pathogen-specific immune response takes days or weeks to develop since the adaptive immune response relies on clonal expansion from a few pre-existing pathogen-specific lymphocytes (2).

Despite the differences between the innate and the adaptive immunity, they interact closely and synergistically in the host defense. The innate immune system plays a central role in the initiation of inflammatory response that is essential for the mobilization and function of the various effector immune cells. Certain innate immune effector cells, such as dendritic cells and macrophages also serve as antigen-presenting cells. Therefore, they play a central role in mounting an efficient adaptive immune response (22). A major function of the innate immune cells during microbial infection is the production of inflammatory mediators such as cytokines, chemokines, nitric oxide, and reactive oxygen species (2). Cytokines and chemokines are essential for the
mobilization of leukocytes to the site of infection, the initiation of the adaptive immune response, and the initiation of acute phase response. The reactive nitrogen and oxygen species are crucial for the killing of the invading pathogens, particularly the pathogen particles engulfed by phagocytes.

B- and T-lymphocytes can strengthen the innate immune cell functions by secreting a variety of cytokines and pathogen-specific antibodies to facilitate the antimicrobial function of innate immune effector cells (23). Antibodies circulate in body fluids where they encounter pathogens and bind to intact pathogen components, targeting them for complement reaction and phagocytosis. On the other hand, T-cell receptors can only bind short peptides that have been assembled into a complex with a membrane glycoprotein called a major histocompatibility complex (MHC) molecule. MHC class I molecules present peptides generated by intracellular pathogens (e.g. viruses) to CD8+ cytotoxic T-cells. MHC class II molecules present peptides generated through digestion of extracellular pathogens in lysosomes to CD4+ helper T-cells (1).

CD4+ T-cells are classified into Th-1 and Th-2 cells depending on the cells they interact with and the cytokines they release. CD4+ T helper (Th)-1 cells primarily produce TNF-α and IFN-γ, which are pro-inflammatory cytokines and promote cellular immunity. In contrast Th-2 cells secrete a different set of cytokines, primarily IL-4, IL-5, and IL-10, which are anti-inflammatory cytokines and promote humoral immunity (24-26). Th-1 and Th-2 responses are mutually inhibitory; therefore, IL-12 and IFN-γ inhibit Th-2 cell differentiation, while IL-4 and IL-10 inhibit Th-1 cell activities.

During the past few years, two new lineages of CD4+ T helper cells, designated as regulatory T cells (Treg) and Th-17 cells have been discovered. Remarkable progress has
been made to characterize these new lineages of helper T cells. Naive CD$_4^+$ T cells activated in the presence of TGF-β and IL-2 upregulate the expression of the transcription factor Foxp3, and develop into ‘inducible’ T regulatory cells (Treg cells), which suppress immune responses (27). On the other hand, TGF-β and IL-6 activate the transcription factors RORγt and STAT3 causing CD$_4^+$ T cells to differentiate into IL-17-producing inflammatory T$_{h}$-17 cells (27).

**The Effect of Cytokines on the Immune System:** The term “cytokine” encompasses a large and diverse family of biologically active polypeptides that are produced by both innate and adaptive immune cells (1). Depending on the location of target cells, the action of cytokines may be autocrine, paracrine, and endocrine. The innate immune system produces various cytokines, chemokines and other inflammatory and anti-inflammatory mediators in response to microbial infection (3). Cytokines play a crucial role in the functioning of the immune system (2). The pro-inflammatory cytokines are not only crucial for the recruitment of immune effector cells such as neutrophils and lymphocytes to the infection site, but also pivotal for the initiation of the adaptive immunity. Cytokines control the proliferation, differentiation, activation of immune effector cells, as well as homeostasis within the immune system (1). Moreover, the pro-inflammatory cytokine IL-6 is pivotal for the initiation of the acute-phase response in the liver. The production of many inflammatory mediators, such as TNF-α, IL-1β and IL-6, as well as prostaglandin and nitric oxide (synthesized by cyclooxygenase (COX)-2 and inducible nitric-oxide synthase (iNOS), respectively), are positively regulated by MAP kinases.
Perhaps reflecting the importance for the fine-tuning of the inflammatory responses, the immune system also produces a number of anti-inflammatory cytokines, including IL-10, TGF-β, and IL-1ra. These anti-inflammatory cytokines are crucial in the resolution of the immune responses, thus ensuring the orchestration of an effective immune response for the elimination of the pathogenic organisms yet avoiding self-destruction. IL-10 is one of the most potent anti-inflammatory cytokines that play an important role in modulating the immune response (28). Numerous reports have consistently demonstrated that the primary biological function of IL-10 is limiting and terminating the inflammatory responses. IL-10 can block the secretion of a large number of pro-inflammatory cytokines. IL-10 knockout mice exhibited a variety of inflammatory diseases associated with enhanced Th-1 responses, including colitis, allergic inflammatory disorders, and increased susceptibility to endotoxic shock (29-31).

1.2 Disorders of the Immune System
The immune system is an amazingly effective structure that incorporates specificity, inducibility, and adaptation of the adaptive immunity into a broad generic anti-microbial innate immune defense mechanism. However, failures of host defense do occur, and can be divided into three broad categories: immunodeficiencies, hypersensitivities, and autoimmunity.

Immunodeficiency diseases are a group of inherited or acquired disorders in which some part(s) of host defense are either defective or absent (1). Hypersensitivity reactions are immune responses to innocuous antigens that lead to symptomatic reactions upon re-exposure (1). They are divided into four classes (Type
I – IV) based on the mechanisms involved and the time course of the hypersensitive reaction. Type I hypersensitivity is an immediate or anaphylactic reaction, often associated with allergy and is mediated by IgE released from mast cells and basophils. Type II hypersensitivity reactions involve IgG antibodies against cell-surface or matrix antigens. Type III hypersensitivity reactions are triggered by the deposit of immune complexes in various tissues (1). Type IV hypersensitivity is known as a delayed type hypersensitivity which usually takes between two and three days to develop and are mediated by effector T cells.

Overactive immune responses are at the other end of the immune dysfunction spectrum, and include autoimmune diseases. Here, the immune system fails to adequately distinguish between self and non-self, and attacks various cells and organs of the host (32). Deregulation of the immune system and excessive production of pro-inflammatory cytokines including TNF-α, IL-1β, IL-6, and IL-8 has been associated with various autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease, psoriasis, and septic shock (33, 34).

*Inflammatory bowel disease:* Crohn’s disease and ulcerative colitis, the two main subtypes of inflammatory bowel disease (IBD), are chronic, relapsing inflammatory disorders of the gastrointestinal tract (35). There is strong evidence to support that IBD is caused by dysregulation of the normally well-controlled immune response to indigenous bacterial flora and other luminal antigens in genetically susceptible individuals (35). The peak age of IBD onset is in the second to fourth decades of life (35). There are approximately 1-1.4 million cases of IBD in the United States (36). The total estimated
annual cost of IBD in the US is approximately $6.3 billion, including both medical expenses (hospitalizations, medications, and surgery) and loss of labor force (36).

Crohn’s disease can be distinguished from ulcerative colitis in that the inflammation associated with Crohn’s disease is transmural and often discontinuous (35). On the other hand, the inflammatory changes of ulcerative colitis usually involve only the superficial mucosal and submucosal layers of the intestinal wall (35). Additionally, ulcerative colitis causes inflammation only in the colon (colitis) and/or the rectum (proctitis), while Crohn's disease may cause inflammation in the colon, rectum, small intestine (jejunum and ileum), and occasionally even the stomach, mouth, and esophagus. Up to 25% of IBD patients will develop extra-intestinal symptoms such as eye, skin, liver disease and arthalgias. IBD patients are also more likely to have other chronic inflammatory diseases, particularly primary sclerosing cholangitis, ankylosing spondylitis, and psoriasis (35).

The pathogenesis of IBD is characterized by an imbalanced activation of Th-1 and Th-2 cells (37). Ulcerative colitis seems to exhibit a Th-2 cytokine profile, and Crohn’s disease appears to primarily exhibit a Th-1 profile (38). Currently, there is no medication that can cure IBD. The goals of current treatments are to induce and maintain remissions, minimize side effects of treatment, and improve the quality of life. Long-term treatment with anti-inflammatory drugs such as aminosalicylates, corticosteroids and immunosuppressants is often accompanied by undesirable and potentially serious side effects (39). Novel biological therapies are targeted to specific pathophysiological processes, offering the potential for better treatment outcomes. TNF-α neutralizing antibodies have shown remarkable efficacy for Crohn’s disease and have been approved
by the FDA for Crohn’s disease. Although not as effective as for Crohn’s disease, a TNF-α-neutralizing monoclonal antibody, Infliximab, also exhibited a clinical response in some patients with moderate-to-severe active ulcerative colitis (40). Furthermore, several new therapeutic strategies are currently being tested in clinical practice. These include recombinant anti-inflammatory cytokines (IL-10 and IL-11), inhibitors of cell adhesion molecules (ICAM), as well as antibodies against pro-inflammatory cytokines (IFN-γ, IL-6 and IL-12) or their receptors (TNFR, IL-6R) (41, 42).

1.3 Immunomodulatory Agents: Due to the pivotal role of pro-inflammatory cytokines in the pathogenesis of a number of inflammatory/autoimmune diseases, substantial efforts have been devoted over the years to modulate the immune system and signaling pathways that control cytokine synthesis. The main goal of such approaches is to restrain cytokine production, to stop the progress of autoimmune disease, and more desirably to reverse the disease process. Certain characteristics are required for a successful immunomodulatory agent applicable in a clinical setting: specificity (only targeting the intended target ideally in a given disease-relevant tissue thus limiting the unintended side-effects), reversibility (temporary effect), and availability of a wide therapeutic window (i.e. the wider the window between the toxic dose (TD50) and the effective dose (ED50), the safer the drug).

*Tripterygium wilfordii Hook F.* (TWFH), is a perennial vine that grows in southern China (43). TWFH is referred to as Lei Gong Teng (Thunder God Vine) in Chinese. Extracts of TWFH have been used in traditional Chinese medicine for centuries for the treatment of immune inflammatory diseases including rheumatoid arthritis, systemic
lupus erythematosus, nephritis, and asthma (44). The root of the plant contains most of the active ingredients. A recent clinical trial sponsored by the National Institutes of Health has demonstrated that extracts of TwHF exhibit remarkable efficacy in rheumatoid arthritis (45, 46). A subsequently published double-blind and placebo-controlled study further confirmed the efficacy and safety of this therapeutic regimen. Characterization of the compounds present in the extracts of Tripterygium identified triptolide (C_{20}H_{24}O_{6}), a diterpenoid triepoxide, as responsible for most of the immunosuppressive, anti-inflammatory and anti-proliferative effects observed in vitro and in vivo (47).

Triptolide has a broad effect on the function of immune effector cells. It has been shown that triptolide inhibits T cell proliferation and blocks the induction of IL-2, IL-4, and IFN-γ (48). Triptolide and TwHF extracts can inhibit the expression of various pro-inflammatory cytokines and adhesion molecules (48). TwHF extracts also inhibit B cell proliferation and immunoglobulin production in response to Staphylococcus aureus (48). Triptolide also attenuates dendritic cell differentiation, maturation, and proliferation (49). TwHF extracts have shown impressive efficacy in a number of animal models of inflammatory and immune-related diseases. TwHF extracts were effective in treating collagen-induced arthritis. The extracts also prolonged the survival of organ transplant and prevented graft-versus host disease (48). In addition to the anti-inflammatory and immune-suppressive effects, triptolide has also demonstrated a remarkable anti-leukemic effect. It has been shown that triptolide induces apoptosis of various tumor cells (48). Despite the promising efficacy of triptolide, its exact mechanism of action remains unclear.
1.4 Mitogen-Activated Protein Kinase Cascades

Mitogen-activated protein kinases (MAPKs) are a group of serine/threonine protein kinases which are highly conserved across eukaryotic species (50). MAPKs play an important role in a variety of cellular processes, such as proliferation, stress response, apoptosis, and immune defense. In multicellular organisms, MAPKs are essential for cellular differentiation, development, memory, learning and secretion of paracrine and autocrine factors (51, 52). Three well-defined MAPK pathways are present in mammalian cells: the extracellular-signal-regulated kinase (ERK) pathway, the Jun N-terminal kinase (JNK) pathway, and the p38 pathway (52). The signals leading to MAPK activation are usually initiated on the cell surface, mainly by various membrane-bound receptors (51, 53). MAPK pathways are activated through a cascade of sequential phosphorylation events, starting with the phosphorylation of MAPK kinases (MAPKKs) at two serine residues by MAPK kinase kinases (MAPKKKs) (52). The activated MAPKKs then phosphorylate MAPKs at the adjacent threonine and tyrosine residues (54). Phosphorylation of these residues on MAPKs results in a substantial conformational change of the protein which increases substrate accessibility and enhances catalysis (55). Activated MAPKs can then phosphorylate a wide array of downstream targets, including protein kinases and transcription factors such as AP-1 that facilitate the transcription of MAPK-regulated genes (56, 57). In addition to transcriptional regulation, MAPKs can regulate the expression of its target genes by altering mRNA stability, transport, and translation (51). The MAPK signaling cascades are not only involved in normal cellular processes, but have also been implicated in the
pathology of many diseases, including cancer (52), atherosclerosis (58), diabetes (59), arthritis (60) and septic shock (61, 62).

MAPK pathways can be modulated by various mechanisms at almost every step of the pathway. These mechanisms include receptor desensitization, dissociation of signaling complexes from receptors as well as deactivation of pathway mediators (50). Since MAPK pathways are activated through phosphorylation, dephosphorylation of kinases, which is mediated by phosphatases, is likely to be one of the most energy-efficient means of deactivation (50). A number of protein phosphatases have been shown to negatively regulate MAPK pathways, such as tyrosine, serine/threonine and dual-specificity MAPK phosphatases (MKPs). Because MKPs dephosphorylate both phosphotyrosine and phosphothreonine residues on activated MAPKs; they are also known as dual specificity protein phosphatases (DUSPs).

1.5 Mitogen-Activated Protein Kinase Phosphatases (MKPs):

Structural features of the MKP family: At least ten MKPs have been identified in mammalian cells so far. Structurally, all MKPs have a highly conserved C-terminal catalytic domain and a less conserved N-terminal region that engages MAPKs (50). The N-terminal region of all MKPs contains a MAPK-interacting domain with the consensus motif ψψXRRψXXG (where ψ represents a hydrophobic residue and X is any amino acid), which is flanked by two CDC25 homology-2 domains (63). The two arginine residues are crucial in the interaction with MAPKs. The exact function of the CDC25-homology-2 domains is unclear, but it has been speculated that these domains might participate in the interaction between MKPs and their cognate MAPKs, thereby further
refining the substrate specificity (63). The cysteine, aspartic acid, and arginine residues in the phosphatase catalytic site at the C-terminal region are essential for enzymatic catalysis. The cysteine residue is required for the nucleophilic attack of the phosphorus on the active MAPK thus forming a thiol-phosphate intermediate during catalysis. The conserved arginine residue interacts with the phosphate group of the MAPK phosphothreonine or phosphotyrosine, and facilitates transition-state stabilization. The aspartic acid residue enhances catalysis by functioning as a general acid which protonates the leaving oxygen group (50).

Subcellular localization: MKPs can be broadly divided into two distinct groups based on their subcellular localization and patterns of transcriptional regulation (50). The first group is primarily localized in the nuclear compartment, is encoded by immediate-early genes, and includes MKP-1 (63-68), MKP-2 (69-71), DUSP2 (PAC1) (69), and DUSP5 (B23) (63, 72). Because these MKPs are rapidly induced by many of the same stimuli that activate MAPKs, for example growth factors and stress, it has been proposed that these MKPs function as a feedback control mechanism for MAPK signaling (68, 73). Since they are localized at the nucleus, these MKPs are thought to play an important role in the deactivation of the transcription factors that are positively regulated by MAPKs (73, 74). The second group of MKPs is not encoded by immediate-early genes, and is either primarily localized in the cytoplasm such as MKP-3 (DUSP6) (75-77), MKP-7 (DUSP16) (78, 79), and MKP-X (DUSP7) (77, 80, 81) or localized in both the cytosolic and nuclear compartments such as HVH5 (DUSP8) (82-84), MKP-4 (DUSP9) (85, 86), and MKP-5 (DUSP10) (87, 88).
Substrate specificity and tissue distribution: In comparison to other protein phosphatases, MKPs show great specificity for the members of the MAPK subfamily (50). However, among these distinct members, there are significant differences in their substrate preferences. For example, MKP-1 shows preference for p38 and JNK (63-68). MKP-2 prefers ERK and JNK (69-71). The preferred substrate for MKP-3, MKP-X, MKP-4, and DUSP5 is ERK (63, 72, 75-77, 80, 81, 85, 86). DUSP8, MKP-5 and MKP-7 prefer JNK and p38 (75-79, 82-84).

Since DUSPs regulate specific cellular responses in certain cell types, DUSPs show different tissue distribution patterns (89). For example, DUSP2 is enriched in hematopoietic cells (90-92). DUSP8 is expressed mainly in brain, heart and skeletal muscle (83). DUSP10 is ubiquitously expressed but is more abundant in cerebellum, skeletal muscle, and bone marrow and is transcriptionally regulated in macrophages (87, 93). DUSP9 is present in placenta, kidney and embryonic liver (86, 94). Furthermore, DUSPs exhibit different expression pattern in various immune cells (89). For instance, MKP-1 is highly expressed in neutrophils, macrophages and B cells, but absent from T-helper cells (89, 90, 95, 96). DUSP2 is absent from non-activated leukocytes but is highly expressed in activated cells, mainly mast cells, neutrophils, and B cells (89). DUSP9 is present in leukocytes, DUSP10 is found in mast cells, dendritic cells and Th-1 cells (89, 90, 95, 96).

MKP-1 is the archetype of the MKP family. Given the pivotal role of p38 and JNK in the regulation of cytokine biosynthesis and the substrate preference of MKP-1 for these two MAPKs, it was expected that MKP1 might play an important role in the control of cytokine biosynthesis (97). Chen and colleagues used immortalized macrophages
RAW 264.7 cells) as a model system to understand the role of MKP-1 in the regulation of cytokine expression in innate immune cells during bacterial infection. They reported that RAW264.7 cell stimulation with LPS resulted in a robust but transient activation of JNK and p38 (97). The kinetics of p38 and JNK inactivation correlated with the accumulation of MKP-1 protein (97). Similarly, stimulation of RAW264.7 cells with peptidoglycan, another bacterial cell wall component, also elicited a transient activation of p38 and JNK which promptly subsided as MKP-1 levels increased (98). These results emphasize the central role of MKP-1 in the inactivation of p38 and JNK in these cells (99).

In principle, all aspects of the immune response that are regulated by MAPKs are potentially affected by MKP proteins. It has been demonstrated that modest levels of ectopic MKP-1 expression in RAW264.7 cells shortened the window of p38 and JNK activation in LPS-stimulated cells, and dramatically attenuated the production of both TNF-α and IL-6 (97, 98, 100). These studies established the concept that MKP-1 is an important negative regulator of innate immune response. Quiescent innate immune cells express very low basal levels of MKP-1 allowing for a window of robust inflammatory response for cytokine production (97-100). However, the rapid induction of MKP-1 allows the system to tune down the inflammatory response, thus achieving homeostasis. Therefore, by limiting the strength and duration of p38 and JNK activation, MKP-1 controls the production of inflammatory cytokines (99). In this sense, MKP-1 serves as a control mechanism to prevent the over-reaction of the innate immune system.

**Regulation of MKPs:** The MKP activity can be regulated by at least three basic mechanisms: transcriptional induction, phosphorylation-mediated changes in protein
stability, and catalytic activation (73). Not all MKPs are regulated by all three mechanisms; some MKPs are inducible, some are stabilized or destabilized after phosphorylation, and others show catalytic activation (50).

The transcription of many MKPs can be induced in response to extracellular stimuli (50). Generally, the expression of the nuclear MKPs (MKP-1, MKP-2, DUSP2 and HVH3) is induced in a robust manner shortly after extracellular stimuli, such as growth factors and cellular stress (73). On the other hand, induction of the cytosolic MKPs is generally less robust, and occurs with slower kinetics (73). Many MKP proteins, including MKP-1, MKP-2, MKP-3 and MKP-7, are post-translationally regulated through phosphorylation (50). Although phosphorylation is not needed for the activation of MKPs, it does alter their stability (101). It has been demonstrated that when MKP-1 is phosphorylated by ERK the half-life of MKP-1 is increased by 2-3-fold (101, 102). This increase in half-life leads to a greater intracellular accumulation of MKP-1 and therefore greater MKP-1 activity. A similar finding has been reported for MKP-7 (103). However, when ERK phosphorylates MKP-3 (an ERK-specific MKP), the MKP-3 degradation is accelerated (104). This leads to less MKP-3 accumulation in the cell, thereby decreasing its effect. Therefore, it appears that MAPKs can either stabilize or destabilize MKPs through phosphorylation. Such phosphorylation-mediated alterations in MKP stability might represent an important crosstalk mechanism between MAPK subfamilies. Finally, the catalytic activities of several MKPs can be considerably enhanced through the interaction with their substrate MAPKs. For instance, the interaction of MKP-3 with its specific substrate ERK2 enhances the phosphatase activity of MKP-3 by approximately 30-fold (105). It has been demonstrated that the crucial
aspartic acid residue in the MKP-3 catalytic domain is nearly 10 Å away from the nucleophilic cysteine and arginine residues at the catalytic site (106), which indicates that substrate-binding-induced catalytic activation of MKP-3 might be due to movement of the aspartic acid residue closer to the catalytic site. Movement of the aspartic acid residue towards the active site allows the aspartic acid to serve as a general acid, thereby facilitating dephosphorylation (106). The complex regulation of these phosphatases, together with their characteristic subcellular localization and substrate preferences, offers a flexible and accurate mechanism that can counterbalance the actions of the very diverse and multifunctional MAPK family (50).

**Study Goals:** The main goals of the studies presented in this dissertation are to understand the mechanisms of regulation of the inflammatory response by two different immune modulators: triptolide, a small molecule isolated from an anti-rheumatic herb in Chinese medicine, *T. wilfordii Hook F.* and MKP-1, an endogenous protein phosphatase. The studies presented in chapter 2 aim at deciphering the anti-inflammatory inflammatory mechanisms of triptolide by investigating its effect on the global gene expression patterns of macrophages treated with LPS. These studies might offer an explanation for the therapeutic mechanisms of *T. wilfordii Hook F.* The goal of the studies proposed in chapter 3 is to understand the basic mechanisms by which MKP-1 regulates the innate immune response and its effect on cytokine and iNOS mRNA stability. The aim of the studies presented in chapter 4 is to understand the role of MKP-1 in the regulation of mucosal immune responses by studying the effect of *Mkp-1* deletion in an *Il-10* knockout mouse model of IBD.
CHAPTER 2

REGULATION OF THE INFLAMMATORY RESPONSE BY TRIPTOLIDE, A DITERPENE TRIEPOXIDE

2.1 Introduction

The herb *T. wilfordii Hook F.*, which is known as “Lei Gong Teng” in Chinese or “Thunder god vine”, has been used for centuries in traditional Chinese medicine to treat rheumatoid arthritis (48). Although much of the clinical experience with this herb comes from uncontrolled studies and anecdotal reports, recent randomized double blind, placebo controlled clinical trials have confirmed the efficacy of the extracts of *T. wilfordii Hook F.* in the treatment of rheumatoid arthritis (45, 46, 107). In addition to rheumatoid arthritis, extracts of *T. wilfordii Hook F.* have also shown efficacy in prolonging allograft survival, as well as treating several other autoimmune and inflammatory diseases including immune complex nephritis and systemic lupus erythematosus (47, 108). Despite the remarkable clinical efficacy, the therapeutic mechanisms of *T. wilfordii Hook F.* extracts remain elusive. In addition to therapeutic properties, *T. wilfordii Hook F.* also exhibits a strong cellular toxicity (109, 110). In fact, another name for *T. wilfordii Hook F.* in Chinese is “seven steps to death”, vividly describing its life-threatening severe
toxicity (111, 112). Because of its severe toxicity, widespread medical application of this herb has been prohibited. Thus, identification of the active ingredients of this plant and understanding of the mode of action of these ingredients may facilitate the development of drugs that are highly efficacious but devoid of significant toxicity.

The extracts of *T. wilfordii* Hook F. contain more than 70 compounds including diterpenoids, triterpenoids, sesquiterpenoids, β-sitosterol, dulcitol, and glycosides (48). Triptolide (C_{20}H_{24}O_{6}), a diterpene triepoxide, is a major component of *T. wilfordii* Hook *F.* extracts, which has been shown to possess potent anti-inflammatory and immunosuppressive properties (48) (Figure 2.1A). For example, triptolide has been shown to inhibit the expression of IL-2 and IFN-γ in T-cells (47, 113). Moreover, it has been demonstrated that triptolide promotes apoptosis of T lymphocytes and dendritic cells (108, 114). In addition to the anti-inflammatory and immunosuppressive activities, triptolide also exhibits potent anti-tumor and anti-leukemic activities (108, 115, 116). Several studies have suggested that triptolide exerts its therapeutic activities by inhibiting the activity of several transcription factors, including NF-κB, AP-1, and NF-AT (115, 117). In the present study, we examined the effects of triptolide on the inflammatory response of innate immune effector cells. cDNA array analysis revealed that triptolide acts as a selective transcriptional blocker predominantly affecting genes involved in immune response. We also investigated the molecular mechanisms mediating the inhibition of cytokine expression by triptolide and demonstrated that triptolide inhibits gene transcription mediated by a number of transcription factors including NF-κB, VP-16, and heat shock factor (HSF)-1 without affecting the transcription machinery. We also explored the role of the hydroxyl group of triptolide in its pro-apoptotic, and anti-
inflammatory activities. We found that substitution of the hydroxyl with an acetyl group abolished both the apoptosis-promoting and the anti-inflammatory effects of triptolide.

2.2 Materials and Methods

Animals – Pathogen-free female C57BL6 mice were maintained on Harlan Teklad irradiated diet (Harlan) at 24°C with relative humidity between 30 and 70% on a 12-h day-night cycle. The experimental protocols were approved by the Institutional Animal Care and Use Committee of The Research Institute at Nationwide Children’s Hospital.

Reagents – LPS (Escherichia coli 055:B5), tetracycline, and triptolide were purchased from Calbiochem (La Jolla, CA). Triptolide was dissolved in DMSO and added to the culture medium. Peptidoglycan (PepG), isolated from S. aureus (Sigma), was dissolved in PBS through sonication and added to the culture medium to the indicated concentrations. CpG DNA (ODN 1826) was purchased from InvivoGen (San Diego, CA).

Cell Culture – Thioglycollate-elicited peritoneal macrophages were isolated from female C57BL6 mice by peritoneal lavage, and cultured at 37°C in RPMI 1640 medium (Invitrogen, San Diego, CA) containing 5% fetal bovine serum (HyClone, Logan, UT) as described previously (98). RAW264.7 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO₂. The CHO-AA8-Luc cells, purchased from Clontech (Mount View, CA) were cultured in Alpha Minimum Essential Medium
(Cellgro, Herndon, VA) containing 10% Tet-approved fetal bovine serum (Clontech), 4 mM glutamine, 1% penicillin/streptomycin, and 100 μg/ml G418. The CHO-AA8-Luc cells harbor a stable luciferase reporter whose expression is under the control of a Tet-Off system (Clontech). Reporter gene transcription was turned off by the addition of tetracycline (2 μg/ml), and turned on by tetracycline withdrawal. Heat shock treatment of RAW264.7 cells were carried out by immersing cells in a 43°C water bath for 1 h.

HeLa (CCL-2) cells were purchased from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) with 4 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, and 10% fetal bovine serum (HyClone, Logan, UT, USA) at 37°C in a humidified atmosphere containing 5% CO2.

cDNA Microarray Analysis – RAW264.7 cells were pre-treated with 50 nM triptolide or vehicle for 30 min, and then stimulated with 100 ng/ml LPS for 4 h. Total RNA was purified using Qiagen RNeasy kit (Qiagen, Valecia, CA). RNA integrity was confirmed with a Bioanalyzer 2100 capillary electrophoresis system and a Degradometer software (118). Labeled cDNA probes were synthesized using 20 ng total RNA and a SPIA Biotin System (119). Labeled cDNA was hybridized to 430A2.0 mouse GeneChips (Affymetrix, Santa Clara, CA) according to manufacturer’s recommendations. The 430A2.0 mouse GeneChip contained sequences representing over 14,000 well defined gene transcripts. Scanned images (DAT files) were converted into CEL files using GCOS software (Affymetrix). Gene expression levels were calculated using RMAExpress (120). Differentially expressed genes were analyzed for over-represented
biological themes (Gene Ontology categories) using EASE software (121). Cluster analyses were performed on two subsets of genes, first those that are up-regulated 5-fold by LPS, and second those that are up-regulated 1-5-fold by LPS and also down regulated at least 50% by triptolide.

Western blot analysis – Western blot analysis was carried out as previously described (97, 98, 100, 122). A rabbit polyclonal antibody against mouse IL-1β was purchased from Chemicon (Temecula, CA). The antibodies against the p50 subunit of NF-κB, IKK, and IκBα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal IKK antibody recognizes both IKKα and IKKβ. The rabbit polyclonal antibody against the p65 subunit of NF-κB was a generous gift from Dr. Dennis Guttridge at the Ohio State University. The polyclonal antibody against phospho-IκBα (p-Ser 32) and an IκBα monoclonal antibody were purchased from Cell Signaling (Danvers, MA). To normalize for protein loading, blots were stripped and probed with a β-actin monoclonal antibody (Sigma, St. Louis, MO).

Northern blotting – Total RNA was isolated from cells using STAT-60 (Tel-Test, Friendswood, TX). Northern blot analysis was carried out using mouse IL-1β, TNF-α, luciferase, and Hsp70 cDNA probes as described previously (123, 124). The membrane was stripped and reprobed with 18S rRNA for normalizing RNA loading.

Northern blotting was also used to assess miR-155 expression. RAW264.7 cells were pretreated with various doses of triptolide for 30 minutes followed by stimulation
with LPS (100 ng/ml) for 4 h. Total RNA harvested using TRIzol reagent (Invitrogen, San Diego, CA) was resolved by electrophoresis on a 12% denaturing urea polyacrylamide gel. RNA was transferred onto a Hybond N+ membrane (Amersham, Piscataway, NJ), and crosslinked to the membrane using UV radiation. The miR-155 and U6 probes were labeled with [α-32P]dATP using StarFire Nucleic Acid labeling system (IDT DNA, Coralville, IA). Hybridization was carried out essentially as previously described (125). The same membrane was stripped and rehybridized with a probe for the small nuclear RNA U6 for normalizing sample loading.

**ELISA** – RAW264.7 cells or primary peritoneal macrophages were plated into 6-well plates at a density of 3×10^5 cells per well. The next day, cells were pretreated either with triptolide (0.01-1 μM) or with equal volume of vehicle (DMSO) for 30 min, and then stimulated with 100 ng/ml LPS for 6 h. TNF-α and IL-6 concentrations in the media were determined by ELISA as previously described (100).

**Animal studies** – C57BL6 mice were first injected with triptolide intraperitoneally (0.15 or 0.25 mg/kg body weight) or an equal volume of vehicle (DMSO). Thirty min later, these mice were challenged with LPS intraperitoneally at a dose of 5 mg/kg body weight. Mice were euthanized 60 min post LPS challenge, and blood was collected by cardiac puncture. Concentrations of TNF-α and IL-6 in the serum were determined by ELISA as previously described (100).
**Immunofluorescence** – To examine the effect of triptolide on the nuclear translocation of NF-κB p65 subunit, we performed immunofluorescence. RAW264.7 cells were treated with LPS (100 ng/ml) in the presence or absence of triptolide (0.1 μM) for the indicated times, controls were treated with DMSO only. Immunofluorescence was essentially carried out as previously described (100, 126), using a rabbit polyclonal anti-p65 antibody (Santa Cruz) and Alexa488-conjugated goat anti-rabbit IgG secondary antibody (Invitrogen). Finally, the cells were stained with 4,6-diamidino-2-phenylindole (DAPI) to visualize the nuclei and were examined under a Zeiss Axioskop microscope (Carl Zeiss, Inc., New York). The fluorescent images were acquired with identical exposure time for all of the samples. The percentage of cells where p65 was localized in the nuclei was scored from at least 10 fields chosen randomly.

**Electromobility gel-shift assay (EMSA)** – RAW264.7 cells were pre-treated with 0.1 μM triptolide and then stimulated with 100 ng/ml LPS. Nuclear extracts were prepared essentially as previously described (127). Oligonucleotides (5′-AGTTGAGGGGACCTTCCCAGGC-3′ and 5′-GCCTGGGAAAGTCCCCTCAACT-3′) were annealed, labeled with [γ-32P]ATP using T4 polynucleotide kinase, and then purified using Sephadex G-25 spin columns. Nuclear extracts (5 μg protein) were incubated with the 32P-labeled double-stranded oligonucleotides for 30 min in a buffer containing 10 mM Tris-HCl (pH 8.0), 0.1% Triton X-100, 12.5% glycerol, 150 mM KCl, 1 mM DTT, 0.5 mM EDTA, and 2 μg of poly IC. Samples were resolved on a 5% nondenaturing polyacrylamide gel using 0.5× TBE (25 mM Tris-HCl pH 8.0, 25 mM
boric acid, 0.5 mM EDTA) as running buffer. Gels were dried, and subjected to PhosphoImaging.

**Plasmids** – Site-directed mutagenesis was carried out to remove the DNA region coding for the VP16 transactivation domain in the pTet-off plasmid (Clontech) while creating a unique *Srf*I site. The transactivation domain of NF-κB was amplified by PCR from a human p65 cDNA with high fidelity *pfu* DNA polymerase, using primers 5'−CCTCAGGCTGTGGCCCCACCTG-3' and 5'−TCATTAGGAGCTGATCTGACTCAG-3'. This blunt-end PCR product was then cloned into the *Srf*I site of the modified pTet-off plasmid. When expressed, this construct results in a fusion protein consisting of the Tet repressor DNA binding protein (TetR) and the transactivation domain of the p65 subunit of NF-κB, referred to hereafter as TetR-p65TA hereafter. The authenticity of the cloning was confirmed by sequencing.

**Transfection and luciferase assays** – To examine the effect of triptolide on reporter transcription mediated by the transactivation domain of NF-κB, RAW264.7 cells were transiently transfected with the plasmid (TetR-p65TA) and the pTRE2-hyg-luc reporter (Clontech), using the polyethyleneimine transfection reagent (Polysciences, Warrington, PA) in the presence of tetracycline. Twenty four h following transfection, cells were fed with medium containing tetracycline, or fed with tetracycline-free medium that contained either 0.1 μM triptolide or vehicle (DMSO) for 24 h. Cells were harvested and luciferase activity measured. Luciferase activity was normalized to lysate protein concentration.
CHO-AA8-Luc cells were grown in the presence of tetracycline until reaching a confluency of ~50%. Then cells were washed with PBS twice and fed with fresh medium with or without tetracycline, in the absence or presence of triptolide. Cells were grown overnight, harvested, and luciferase activity in the lysates was measured using a luminometer with a Luciferase Assay System (Promega, Madison, WI).

*In vitro transcription assay* – Mixtures of transcription factors, DNA templates and $\alpha$-$^{32}$P CTP, were incubated with 100 nM of triptolide or vehicle (DMSO) at 30°C for 60 min. The reactions for the basal level transcription were performed with the transcription kit from ProteinOne (Bethesda, MD) according to the manufacturer’s recommendations. *In vitro* assay for activated transcription was reconstituted with purified transcription factors (TFIID, TFIIA, GAL4-AH, PC4) in the presence of template pG5HM and pMLΔ53 essentially as described (128). RNA was isolated by phenol chloroform extraction, resolved by electrophoresis on a 5% urea-polyacrylamide gel and visualized by autoradiography.

*Synthesis of O-acetylated triptolide* – The reactions that required anhydrous conditions were performed in oven-dried glassware which was cooled under argon. $^1$H and $^{13}$C NMR spectra were performed on 400 MHz or 300 MHz Bruker NMR spectrometer. Unless otherwise indicated, all NMR data were collected at room temperature in CDCl$_3$ with internal CHCl$_3$ as the reference (7.26 ppm for $^1$H and 77.23 ppm for $^{13}$C). Analytical thin-layer chromatography (TLC) was carried out on commercial silica gel 60 plates, 0.25 thickness, with fluorescent indicator (F-254). Visualization was accomplished by UV
light. Acetylated triptolide (designated as TD-101 hereafter, figure 2.1B) was prepared from triptolide by the following procedures. N,N-dimethylaminopyridine (17.8 mg, 0.14 mmol) and acetyl chloride (6.6 μl, 0.091 mmol) were added to a solution of triptolide (5 mg, 0.014 mmol) in CH₂Cl₂ (1 ml) under argon. The mixture was stirred at room temperature for 20 h. The solvent was removed via rotary evaporation and the solid residue was purified by preparative TLC using 1:1 (vol/vol) ethyl acetate/hexanes as eluent. The desired product was obtained as a white solid (4.7 mg, 84% yield): Rf = 0.45; ¹H NMR (400 MHz, CDCl₃) δ 5.08 (s, 1H), 4.67 (s, 2H), 3.82 (d, J = 2.8 Hz, 1H), 3.53 (d, J = 2.8 Hz, 1H), 3.46 (d, J = 5.6 Hz, 1H), 2.71-2.67 (m, 1H), 2.35-2.29 (m, 1H), 2.20-2.13 (m, 2H), 2.18 (s, 3H), 1.94-1.85 (m, 2H), 1.63-1.59 (m, 1H), 1.27-1.18 (m, 1H), 1.06 (s, 3H), 0.96 (d, J = 7.2 Hz, 3H), 0.85 (d, J = 6.8 Hz, 3H).

Cell Proliferation – HeLa cells 1.5×10⁶ cells were initially plated into 100-mm tissue culture dishes. Cells were either treated with increasing concentrations of triptolide or TD-101 for 24 h. Apoptotic cells were determined based on cellular morphology. Cell morphology was documented by photography under a Zeiss Invertoskop 40C inverted microscope (Carl Zeiss, Thornwood, NY).

Statistics – The results from the experiments assessing the effects of triptolide on TNF-α and IL-6 production were analyzed by one-way ANOVA with LSD post hoc test. Statistical analysis was carried out using SPSS statistical software program (SPSS, Chicago, IL). Differences were considered significant when p < 0.05.
2.3 Results

Triptolide selectively inhibits the expression of inflammatory genes including pro-inflammatory cytokines and chemokines – To assess the effect of triptolide on the inflammatory response of innate immune cells, we performed microarray analysis. To this end, RAW264.7 macrophages were pre-treated with 50 nM triptolide or vehicle for 30 min, and then stimulated with LPS for 4 h. Total RNA was harvested and used to produce cDNA probes. Labeled cDNA was hybridized to 430A2.0 mouse GeneChips (Affymetrix), which contained sequences corresponding to over 14,000 well defined gene transcripts. Gene expression levels were calculated using RMAExpress. Differentially expressed genes were analyzed for over-represented biological themes (Gene Ontology categories) using EASE software. LPS treatment resulted in >5-fold induction in the expression of 117 genes (Figure 2.2A). Among these 117 genes induced by LPS, 47 of them were inhibited by 50% or more by triptolide (50 nM). Many of these LPS-induced, triptolide-attenuated genes are involved in immune function/host defense, including pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, colony stimulating factor (CSF)-2 and 3) and chemokines (CCL-1, 2, 5, 7, and 12 as well as CXCL-10 and 11). In addition to these cytokines and chemokines, triptolide also inhibited the expression of several adhesion molecules (laminin γ1, integrin α5, ICAM1) and a number of transcription factors (ATF3, NF-κB, STAT5α), as well as BIC that is the precursor of miR-155. The global effects of triptolide on the expression of genes that are potently induced by LPS (>5-fold) are shown in Figure 2.3.

We also analyzed the effects of triptolide on the expression of genes that are induced less potently by LPS or are relatively insensitive to LPS (1-5-fold induction by
LPS relative to unstimulated control). Total of 10,600 genes fell into this category. Among these genes, the expression levels of 119 genes were decreased by triptolide pretreatment by at least 50% (Figure 2.2B). These results indicate that triptolide does not block global gene expression in a nondiscriminatory manner. Our analysis suggests that triptolide preferentially inhibits genes that are highly inducible by inflammatory stimuli.

**Triptolide inhibits pro-inflammatory cytokine production in LPS-stimulated macrophages** – To validate the results of the cDNA array analysis, we examined the effect of triptolide on the expression of a small set of pro-inflammatory cytokine genes. Since pro-inflammatory cytokines such as TNF-α and IL-1β are critical in the pathogenesis of inflammatory diseases, including rheumatoid arthritis, understanding of the mechanism underlying the inhibitory effect of triptolide on these cytokines may reveal important information on the mode of action of this novel compound. The effect of triptolide on the expression of IL-1β and TNF-α induced by LPS in RAW264.7 cells was examined by Northern blot analysis. While basal IL-1β and TNF-α mRNA levels were undetectable in control cells, these genes were dramatically induced upon LPS stimulation. Treatment with triptolide caused a dose-dependent inhibition in both IL-1β and TNF-α mRNA levels (Figure 2.4A). Substantial inhibition of IL-1β and TNF-α mRNA levels were observed in samples treated with triptolide at concentrations as low as 50 nM.

The effect of triptolide on the induction of these cytokines in primary macrophages was also examined. As in the RAW264.7 cells, the induction of both TNF-α and IL-1β by LPS in primary macrophages was strongly inhibited by triptolide in a
dose-dependent manner (Figure 2.4B). The inhibitory effects of triptolide on these cytokine mRNAs were clearly visible starting at 20 nM.

The effect of triptolide on the production of cytokine proteins was examined by ELISA. LPS potently induced the production of both TNF-α and IL-6 in RAW264.7 macrophages, and triptolide potently inhibited the cytokine production in a dose-dependent manner, with an IC50 of <30 nM for both TNF-α and IL-6 (Figure 2.5A). Impressively, treatment with 50 nM triptolide resulted in a greater than 80% decrease in both TNF-α and IL-6 production in LPS-stimulated cells. Triptolide also potently suppressed LPS-induced IL-1β production in a dose-dependent manner.

Similar to what happened in RAW264.7 cells, triptolide potently inhibited IL-1β production in LPS-stimulated primary macrophages in a dose-dependent manner (Figure 2.5B). Likewise, production of both TNF-α and IL-6 production in primary macrophages were also inhibited by triptolide. Triptolide, at a concentration of 10 nM, inhibited TNF-α production by approximately 80%. Compared to TNF-α, IL-6 production appeared to be less sensitive to triptolide (Figure 2.5B). Triptolide, at concentrations of 10 and 50 nM, inhibited IL-6 production by ~40% and ~80%, respectively.

*Triptolide inhibits pro-inflammatory cytokine production in macrophages stimulated with other TLR ligands* – We then examined the effect of triptolide on inflammatory cytokines induced by bacterial components other than LPS. Peptidoglycan (PepG), is a cell wall component in both Gram-positive and Gram-negative bacteria. CpG sites are regions of DNA where a cytosine nucleotide occurs next to a guanine nucleotide in the linear
sequence of bases along its length. Cytosines in CpG dinucleotides are methylated by DNA methyltransferases in many eukaryotic organisms to form 5-methylcytosine. Mammals methylate 70%-80% of CpG cytosines (129). Unmethylated CpG sites derived from intracellular bacterial DNA from pathogens can be detected by macrophages in humans. Therefore, unmethylated CpG DNA is often used to mimic unmethylated bacterial DNA to study the immune response to pathogens.

The effect of triptolide on the expression of IL-1β and TNF-α induced by the TLR ligands (PepG, CpG DNA) in RAW264.7 cells was examined by Northern blot analysis. Basal IL-1β and TNF-α mRNA levels were undetectable in control cells. These genes were dramatically induced upon stimulation with PepG or CpG DNA (Figure 2.6). Treatment with triptolide attenuated the induction of both IL-1β and TNF-α mRNA in a dose-dependent manner (Figure 2.6). Significant inhibition of IL-1β and TNF-α mRNA levels were observed in samples treated with triptolide at concentrations as low as 50 nM and 10 nM, respectively.

The effect of triptolide on the induction of these cytokines in primary macrophages was also examined. As in the RAW264.7 cells, the induction of both TNF-α and IL-1β mRNA by PepG and CpG DNA in primary macrophages was strongly inhibited by triptolide in a dose-dependent fashion (Figure 2.7).

The effect of triptolide on the production of cytokine proteins was examined by ELISA. Peptidoglycan and CpG DNA robustly induced the production of both TNF-α and IL-6 in RAW264.7 macrophages, and triptolide potently inhibited the cytokine production in a dose-dependent manner (Figure 2.8). Triptolide also effectively
suppressed PepG- and CpG DNA-induced IL-1β production in a dose-dependent manner (Figure 2.8).

Similar to what was observed in RAW264.7 cells, triptolide potently inhibited TNF-α and IL-6 production in PepG- and CpG DNA-stimulated primary macrophages in a dose-dependent manner (Figure 2.9). IL-1β production in primary macrophages was also strongly inhibited by triptolide in a dose-dependent manner (Figure 2.9). These data suggest that triptolide acts as a potent anti-inflammatory molecule during the innate immune response to various TLR ligands.

Triptolide inhibits the induction of miR-155 in LPS-stimulated macrophages – miR-155 is a key player in inflammatory response, our microarray results showed that the expression of the miR-155 precursor, BIC, was up regulated 7-fold following stimulation with LPS. Triptolide attenuates BIC expression by 80%. To validate these results, RAW264.7 cells were pre-treated cells with triptolide (0.01-0.5 μM) for 30 min followed by stimulation with LPS for 4 h, and miR-155 expression was assessed by Northern blot analysis. LPS treatment resulted in a robust increase in miR-155 level, and miR-155 induction by LPS was attenuated in a dose-dependent manner by triptolide (Figure 2.10).

Triptolide attenuates the inflammatory response in vivo in LPS-challenged mice – To investigate the effects of triptolide on the inflammatory response in vivo, C57BL6 mice were injected intraperitoneally with either triptolide or the vehicle, and then challenged with LPS 30 min later. Blood was harvested 60 min post LPS challenge, and cytokine concentrations in the serum were measured. Challenge of C57BL6 mice with LPS
caused a robust increase in TNF-α production as compared to unchallenged mice (Figure 2.11A). At a dose of 0.15 mg/kg body weight, triptolide decreased blood TNF-α levels by 64%. At a higher dose (0.25 mg/kg body weight), triptolide almost completely abolished TNF-α production in vivo. Likewise, triptolide markedly inhibited the LPS-stimulated increase in blood IL-6 levels in a dose-dependent manner (Figure 2.11B).

*Triptolide has no effect on IκBα degradation and p65 nuclear translocation, and does not affect NF-κB DNA binding activity* – Previously, it has been reported that NF-κB activity is inhibited by triptolide (117). Furthermore, a study by Zhou et al. showed that IκB degradation and NF-κB DNA-binding activity are inhibited by a triptolide derivative (130). To elucidate the mode of action of triptolide, RAW264.7 cells were treated with LPS in the absence or presence of triptolide, and kinetics of IκBα phosphorylation were determined by Western blot analysis using an anti-phospho-IκBα antibody (Figure 2.12A). Upon LPS stimulation, the levels of phospho-IκBα peaked at about 10 min, then rapidly plummeted. The decreases in phospho-IκB levels were associated with decreases in IκBα protein levels, detected by Western blotting using a monoclonal antibody recognizing total IκBα. At 60 min, IκBα protein levels increased again, likely due to de novo protein synthesis. This increase in newly synthesized IκBα coincided with increased phospho-IκBα levels, suggesting IκB kinases remained active at least up to 60 min post LPS stimulation (Figure 2.12A). Importantly, neither the kinetics of IκBα phosphorylation nor IκBα degradation was significantly affected by triptolide. The levels of p65, p50, and IKK proteins did not change regardless of whether the cells were
pretreated with triptolide or not (Figure 2.12A). We then investigated the effect of triptolide on NF-κB translocation to the nucleus, by immunofluorescence staining of the p65 subunit of NF-κB (Figure 2.12B). p65 was almost exclusively localized in the cytoplasm in control cells, and translocated to the nuclei upon LPS stimulation. Triptolide had no effect on the kinetics of nuclear translocation of NF-κB p65 subunit in LPS-stimulated RAW264.7 cells (Figure 2.12B). To examine if triptolide inhibits the DNA binding activity of NF-κB, RAW264.7 cells were pre-treated with triptolide for 30 min then stimulated with LPS. Nuclear extracts were collected at the indicated times and EMSA was performed. LPS stimulation resulted in an increase of NF-κB DNA-binding activity, which was not affected by triptolide (Figure 2.12C).

Triptolide blocks gene transcriptional induction regulated by a variety of transcriptional factors – Previous studies have suggested that triptolide inhibits transactivation of NF-κB at a step after binding to DNA (117). To gain insight into the inhibitory mechanism of NF-κB by triptolide, we examined the effect of triptolide on the activity of the transactivation domain of p65, a subunit of NF-κB, by taking advantage of the Tet-Off system. We modified the Tet-Off system by replacing the VP16 transactivation domain with the transactivation domain of p65. This modification yielded an artificial hybrid transcription factor TetR-p65TA, which consists of the DNA-binding domain of TetR and the transactivation domain of p65. This construct was transfected into RAW264.7 cells together with a tetracycline-regulated luciferase reporter gene (pTRE-Luc), and the effect of triptolide on reporter gene expression was assessed. TetR-p65TA efficiently
turned on the transcription of the luciferase reporter in the absence of tetracycline and triptolide potently inhibited the reporter gene transcription (Figure 2.13A). As a control, we also assessed the effect of triptolide on reporter transcription mediated by the artificial transcription factor TetR-VP16, using a stable cell line (CHO-AA8-Luc) which harbors both the TetR-VP16 and the TRE-Luc reporter constructs. Surprisingly, triptolide showed a dose-dependent inhibition of the luciferase reporter transcription. Triptolide at concentrations of 50-100 nM almost completely abrogated reporter expression elicited by tetracycline withdrawal (Figure 2.13B). Northern blot analysis further confirmed that triptolide, at a concentration of 20 nM, abolished luciferase mRNA expression induced by tetracycline withdrawal (Figure 2.13C).

To explore the specificity of triptolide on the transcriptional induction of endogenous genes, we examined the effect of triptolide on Hsp70, which is induced by heat shock through a completely different transcription factor, heat shock factor (HSF)-1 (131). To this end, RAW264.7 cells were pre-treated with either various doses of triptolide or vehicle (DMSO), and then subjected to heat shock at 43°C for 1 h. Hsp70 mRNA was detected by Northern blot analysis. Hsp70 mRNA in control cells was undetectable, and its mRNA levels increased dramatically after heat shock. Triptolide pre-treatment caused a dose-dependent inhibition of Hsp70 mRNA induction (Figure 2.13D). These studies clearly indicate that triptolide not only inhibits the activity of NF-κB, but also has broad inhibitory effects on gene transcription mediated by other transcription factors.

To elucidate the mechanism by which triptolide blocks gene transcription, we examined the effect of triptolide on gene transcription using an in vitro gene transcription
assay. The reaction mixtures containing RNA polymerase II complex, transcription factors and co-activators were incubated with 100 nM triptolide or DMSO. Basal activity of the transcription machinery was assayed using two plasmid DNA templates: pG5HM and pMLΔ53. Basal transcription requires a number of indispensable transcription factors (TBP, TFIIB, and TFIIE/F/H) while activated transcription relies on activators and cofactors that are not necessary for basal transcription (TFIIA, TFIID, GAL4-AH, and PC4). The pG5HM template contains 5 GAL4 binding sites and can support both basal and activated transcription. However, the pMLΔ53 plasmid has no GAL4 binding sites and can only be transcribed by the basal transcription machinery. As indicated in Figure 2.13E, triptolide at a concentration of 100 nM had little effect on in vitro gene transcription mediated by the basal transcriptional machinery. Likewise, triptolide also did not have an appreciable effect on gene transcription mediated by the GAL4-AH transcriptional factor in vitro. These results suggest that triptolide does not simply block the transcription machinery.

The importance of the hydroxyl group in the activities of triptolide – To understand the structural basis of the triptolide activities, we modified triptolide by acetylating the hydroxyl group at position 14 and refer to the acetyl derivative as TD-101 hereafter (See Figure 2.1 for structures). The effects of this modification on triptolide activity were examined. As demonstrated previously, LPS stimulation of RAW264.7 cells resulted in a robust production of TNF-α (Figure 2.14A). Triptolide strongly inhibited the production of TNF-α, at concentrations as low as 10 nM. In contrast, TD-101 exhibited little inhibition on TNF-α production even at the highest concentrations. Similarly, while
triptolide potently attenuated the production of IL-1β in LPS-stimulated macrophages, with almost complete inhibition observed at 50 nM, TD-101 had no detectable effect on IL-1β (Figure 2.14B).

We also examined the effect of this modification on the apoptosis-promoting activity of triptolide, using HeLa cells as a model system. Unlike triptolide that potently induced HeLa cells to undergo apoptosis at concentrations as low as 20-50 nM, TD-101 exhibited little apoptosis-promoting activity at concentrations up to 200 nM. At 500 nM, TD-101 induced apoptosis in a small number of HeLa cells (Fig. 2.15). Taken together, these results indicate that the hydroxyl group of triptolide plays a critical role in mediating both the apoptosis-promoting and anti-inflammatory activities.

2.4 Discussion

*Anti-inflammatory mechanism of triptolide* – Triptolide has potent anti-inflammatory properties. It has been shown that triptolide inhibits experimental autoimmune uveoretinitis (132) and prolongs allograft survival (43). Moreover, it has been demonstrated that a succinyl derivative of triptolide, PG490-88, can prevent graft-versus-host disease (133). Although the immunosuppressive action of triptolide has been generally attributed to suppression of T-lymphocyte activation (47, 48), recent studies have also provided evidence that triptolide also inhibits innate immune functions. For example, Lu *et al.* have demonstrated that triptolide blocks the production of two chemokines, IL-8 and MCP-1, in cultured human corneal fibroblasts stimulated with proinflammatory cytokines (134). These findings suggest that triptolide may exert its anti-inflammatory effects by limiting the infiltration of neutrophils and monocytes into
the cornea (134). Very recently, Zhu et al. and Chen et al. have shown that triptolide can inhibit the differentiation, maturation, trafficking, and function of dendritic cells, the professional antigen presenting cells (49, 135). Moreover, triptolide has also been shown to suppress CD80 and CD86 expression and block IL-12 production in human monocytic THP-1 cells (136). In the present report, we performed microarray analysis on LPS-treated RAW264.7 cells that have been pretreated with triptolide (Figure 2.2). Our results indicate that triptolide exerts its anti-inflammatory activity through selective transcriptional blockade of genes involved in the immune response (Figure 2.3). These results are in agreement with microarray results reported by Zhao et al. showing that triptolide is not a nonspecific inhibitor of transcription in bronchial epithelial cells (137). The selectivity of transcriptional inhibition by triptolide suggests that it interferes with transcription factors regulating the transcription of a subset of genes, predominantly cytokines and chemokines. We have examined the effect of triptolide on the production of pro-inflammatory cytokines in macrophages, a major source of pro-inflammatory cytokines. We found that triptolide abrogated the production of pro-inflammatory cytokines in response to LPS stimulation both in vitro (Figure 2.5) and in vivo (Figure 2.11). Profound inhibition of pro-inflammatory cytokine expression in response to LPS was observed at concentrations as low as 10-50 nM. The inhibition of cytokine production by triptolide occurs at the mRNA level in both immortalized and primary macrophages (Figure 2.4), suggesting that triptolide blocks the transcription of pro-inflammatory cytokine genes. Similarly, we have found that triptolide inhibits cytokine production at mRNA and protein levels in response to both TLR2 and TLR9 ligands (Figures 2.6-2.9). This can be explained by the fact that the bacterial components used in
our experiments share common signaling pathways with LPS. These findings indicate that triptolide has a broad spectrum of anti-inflammatory activity against different bacterial components and its activities are not limited to LPS. These results suggest that inhibition of pro-inflammatory cytokine expression by triptolide may play an important role in the anti-rheumatic/anti-inflammatory properties of *T. wilfordii Hook F*.

_The mechanism by which triptolide inhibits the production of inflammatory cytokines –_ Triptolide has three highly reactive epoxide groups, which are capable of reacting with nucleophilic protein side chains such as those on histidine, cysteine, serine, and aspartic acid. Covalent modification of protein target(s) by an epoxide group(s) of triptolide is consistent with the exceptional potency of this compound. Very recently, it has been reported that triptolide binding to HeLa cells was saturable, reversible, and primarily localized to cell membrane proteins with molecular mass ranging from 75 to greater than 250 kDa (138). The fact that triptolide binding to proteins can be detected after separation on denaturing SDS-polyacrylamide gels strongly argues that triptolide interacts with its targets through covalent bonds. Due to the high reactivity of the epoxide rings, triptolide is expected to react with a variety of intracellular nucleophiles such as glutathione and have a relatively short half-life. This may explain the observed “reversibility” of the compound (138, 139), although it is also possible that the covalent linkage between triptolide and its protein target(s) is chemically reversible.

The observation that the transcription of several inflammatory cytokines is potently inhibited by triptolide suggests that triptolide may either disrupt the signaling process leading to cytokine transcription or directly interfere with the transcriptional
process. NF-κB is a transcription factor that plays a critical role in the transcriptional induction of many inflammatory cytokines (140, 141). Since it has been proposed that triptolide inhibits NF-κB activity at a step after NF-κB binding to DNA (117), we examined the effects of triptolide on the NF-κB pathway. We found that triptolide pretreatment neither affects the phosphorylation-mediated degradation of IκBα after LPS stimulation, nor decreases the DNA-binding activity of NF-κB in cell nuclear extracts (Figure 2.12). These observations are in agreement with the findings that DNA binding activity of NF-κB is not affected by triptolide (117, 137). However, our findings contradict recent reports that triptolide inhibited LPS-induced increases in the DNA-binding activity of NF-κB in primary macrophages and RAW264.7 cells (142, 143). The exact reasons for these discrepancies are unclear, but may represent differences in experimental methodologies.

Several earlier studies have examined the effect of triptolide on the regulation and activity of NF-κB (117, 144). Using a luciferase reporter containing a NF-κB-binding element, Qiu et al. have shown that triptolide potently abolishes the reporter gene transcription elicited by TNF-α. Likewise, both Zhao et al. and Lee et al. have found that triptolide potently inhibits NF-κB-dependent reporter transcription induced by PMA or the inflammatory cytokines, TNF-α and IL-1β (137, 144). More recently, Leuenroth and Crews have demonstrated that triptolide blocks NF-κB-dependent luciferase reporter transcription at a concentration greater than 50 nM (138). Using a GAL4-NF-κB hybrid transcription factor construct together with a GAL4-dependent luciferase reporter system, Qiu et al. have shown that triptolide abolishes transcription activation mediated by the
NF-κB transactivation domain (117). These investigators have postulated that triptolide blocks NF-κB-mediated transcription by interfering with p65 modification or the recruitment of a transcriptional cofactor (117, 144). In the present study, we have investigated the effects of triptolide on the transcription-enhancing property of the NF-κB transactivation domain, using a modified Tet-Off system (Figure 2.13A). Unlike the original Tet-Off system that uses the transactivation domain of the viral VP16 protein, the modified system described herein relies on the transactivation domain of NF-κB. Compared to the GAL4-NF-κB-coupled luciferase reporter system used by Qiu et al. (117), which is constitutively active when expressed, reporter gene expression driven by the NF-κB-based Tet-Off system described herein has the obvious advantage of being expressed only after tetracycline withdrawal. With this system, we found that triptolide, at a concentration of 100 nM, inhibited NF-κB-driven reporter gene transcription by ~75% (Figure 2.13A).

Surprisingly, triptolide also dramatically inhibited the VP16-driven luciferase reporter activity (Figure 2.13B). To rule out the possibility that triptolide may directly react with luciferase and abolish its enzymatic activity, we performed Northern blot analysis (Figure 2.13C). Our results indicate that luciferase transcription was completely abolished by triptolide at concentrations as low as 20 nM (Figure 2.13C). These results clearly indicate that the inhibition of gene transcription by triptolide is not only limited to genes transcriptionally regulated by NF-κB. This notion is further supported by the finding that induction of Hsp70 in response to heat shock, mediated by a distinct transcription factor HSF-1, is also strongly inhibited by triptolide (Figure 2.13D).
inhibitory effect of triptolide on Hsp70 induction in HeLa cells has been reported recently (139). Similar to what was observed herein for NF-κB, Westerheide et al. found that triptolide abrogates the transactivation function of HSF-1 without interfering in the early events of trimer formation, hyperphosphorylation, and DNA binding (139). The fact that triptolide blocks the activities of three unrelated transcription factors (NF-κB, VP16, and HSF-1) suggests that triptolide inhibits a common step(s) of the transcriptional processes mediated by these transcription factors. While the mechanisms involved are currently unclear, we can make some speculations. First, it is possible that triptolide poisons the basal transcription machinery. However, we consider this scenario unlikely, since not all genes are inhibited equally by triptolide. Microarray experiments clearly indicate that some genes are more sensitive than others to inhibition by triptolide (Figure 2.2 and 2.3). For example, triptolide significantly inhibits TNF-α expression at concentrations between 20-100 nM. Although MKP-1 is also potently induced by LPS, significant inhibition was only observed at concentrations between 500-1,000 nM (97). Second, a significant inhibition of basal gene transcription by triptolide was not observed in an in vitro assay. While we recognize the limitations of our in vitro transcription assays, we think that triptolide likely only inhibits the transcription mediated by a subset of transcription factors. This notion is consistent with a recent report that triptolide inhibits the interaction between p65 and the transcription coactivator CBP/p300 (145). Indeed, the inhibition of a transcriptional mechanism utilized by a variety of transcription factors may explain the serious toxicity of triptolide and T. wilfordii Hook F. Further characterization of the interaction between triptolide and transcription factors or transcriptional coactivators may not only reveal novel targets for the development of anti-
inflammatory and anti-rheumatic drugs, but also facilitate the development of less toxic triptolide derivatives for therapeutic purposes.

The pivotal role of C-14 hydroxyl in the pharmacological activity of triptolide – Although triptolide represents a promising drug candidate for the treatment of cancer and inflammatory diseases, its application is hampered by its severe toxicity (146, 147). Thus, understanding the structural basis for its therapeutic activity and its cytotoxicity may allow one to synthesize triptolide derivatives or analogues that have an improved therapeutic index. The availability of properly derivatized triptolide (e.g., biotinylated triptolide) may also help identify its cellular target(s). As the first step toward these goals, we examined the possibility of derivatizing triptolide at the hydroxyl group at position 14, the only nucleophilic functional group in the molecule that can be conveniently modified. We found that simple acetylation of the hydroxyl group almost completely abrogated both the anti-inflammatory activity (Figure. 2.14) and the apoptosis-promoting activity of triptolide (Figure 2.15). It has previously been reported that PG490-88, a water-soluble derivative of triptolide created by acylating the C-14 hydroxyl group with a succinyl group retains significant anti-inflammatory activity (148). These apparently conflicting results can be readily explained by the different stabilities of the two acylated derivatives in aqueous solution. In PG490-88, the free carboxylate group of the succinyl moiety promotes the hydrolysis of the ester linkage. Consequently, the succinyl derivative is much less stable than the acetyl derivative, undergoing rapid hydrolysis to give back the active drug, triptolide (John Fidler, personal communication). Hydrolysis of the acetic ester in TD-101 can also take place, but occurs at a much slower
rate. The residual activity of TD-101 at high concentration is most likely due to trace amounts of triptolide derived from the slow hydrolysis of TD-101 (Figures 2.14 and 2.15). These studies demonstrate that a free hydroxyl group at C-14 is critical for triptolide activity. However, a single hydroxyl group is unlikely to provide most of the binding energy to its biological target(s). Thus, a more plausible explanation for the dramatic effect of acylation of C-14 hydroxyl is that the acyl group sterically blocks either the binding of triptolide to its biological target(s) or the covalent reaction between the epoxide group and the protein side chain(s). Inspection of the calculated 3D structure of triptolide indicates that the epoxide between C-12 and C-13 is most exposed and most likely the site of modification. It is likely that nucophile attack takes place from the back side of this epoxide. Since the C-14 hydroxyl is situated on the back side and close to the epoxide, addition of a bulky acyl group is expected to prevent an incoming nucleophile from back attack on the epoxide.
Figure 2.1 The structure of triptolide (A) and its acetyl derivative TD-101 (B).
Figure 2.2 Triptolide selectively inhibits the expression of a subset of genes involved in the immune response. RAW264.7 cells were either treated with vehicle or LPS in the presence or absence of triptolide, and microarray analyses were performed. Heat maps were generated representing cluster analyses of genes exhibiting 5-fold or greater induction by LPS (A) or 1-5-fold induction by LPS but inhibited by triptolide at least 50% (B).
**Figure 2.3** Triptolide potently inhibits the expression of genes that were potently induced by LPS. The graph depicts the effect of triptolide on the genes highly induced (>5-fold) by LPS. Dots above the line represent genes whose expression is enhanced by triptolide. Dots below the line represent genes whose expression is inhibited by triptolide.
**Figure 2.4** Triptolide blocks the induction of pro-inflammatory cytokine mRNA in LPS-stimulated macrophages. RAW264.7 cells (A) and primary peritoneal macrophages (B) were pretreated with indicated doses of triptolide for 30 min, and then stimulated with LPS (100 ng/ml) for 4 h. Total RNA was harvested and analyzed by Northern blotting. The IL-1β and TNF-α mRNA signals were normalized to 18S rRNA signals, and expressed as fold increase relative to control. Numbers below the blots indicate fold of changes in gene expression relative to untreated controls.
Figure 2.4

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- IL-1β

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- 18S

B

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- IL-1β

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- 18S
**Figure 2.5** Triptolide potently inhibits the production of pro-inflammatory cytokine proteins in macrophages treated with LPS. RAW264.7 cells (A) and primary peritoneal macrophages (B) were pretreated with indicated doses of triptolide for 30 min followed by stimulation with 100 ng/ml LPS for 6 h. The negative control was only treated with equal volume of vehicle (DMSO). Positive controls were those first treated with vehicle (DMSO) then stimulated with LPS. Upper panels represent the effect of triptolide on TNF-α and IL-6 secretion, which were assayed by ELISA. Results in the graph represent % relative to the mean value of the positive controls. Data were expressed as means ± S.E. from 3 independent experiments. *, *p*<0.05, compared to the group stimulated with LPS after pretreatment with vehicle. Lower panels demonstrate the effect of triptolide on IL-1β production detected by Western blot analysis. Presented are the representative results of at least three experiments.
Figure 2.5

A

B

Triptolide (µM)

% of control

Triptolide (µM)

% of control

Triptolide

0 0.01 0.02 0.05 0.1 0.2 0.5 1

- IL-1β

- β-actin

LPS - + + + + + + + +

- TNF-α

- IL-6

LPS - + + + + + + + +
**Figure 2.6** Pro-inflammatory cytokine mRNA expression in PepG- and CpG DNA-stimulated macrophages is potently inhibited by triptolide. RAW264.7 cells were pretreated with indicated doses of triptolide for 30 min, and then stimulated with 10 μg/ml PepG (A) or 3 μg/ml CpG DNA (B) for 4 h. Total RNA was harvested and analyzed by Northern blotting. The IL-1β and TNF-α mRNA signals were normalized to 18S rRNA signals, and expressed as fold increase relative to control. Numbers below the blots indicate fold of changes in gene expression relative to untreated controls.
Figure 2.6

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- IL-1β (fold)

1 321 267 235 108 24 3 2 1 2

- TNFα (fold)

1 857 353 282 80 28 5 1 0 0

- 18S

B

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- IL-1β (fold)

1 133 97 81 40 9 1 1

- TNFα (fold)

1 106 48 33 10 3 2

- 18S
Figure 2.7 Induction of pro-inflammatory cytokine mRNA in primary macrophages stimulated with PepG or CpG DNA is inhibited by triptolide. Primary peritoneal macrophages were pretreated with indicated doses of triptolide for 30 min, and then stimulated with either 10 μg/ml PepG (A) or 3 μg/ml CpG DNA (B) for 4 h. Total RNA was harvested and analyzed by Northern blotting. The IL-1β and TNF-α mRNA signals were normalized to 18S rRNA signals, and expressed as fold increase relative to control. Numbers below the blots indicate fold of changes in gene expression relative to untreated controls.
Figure 2.7

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- IL-1β (fold)
- TNFα (fold)
- 18S

B

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- IL-1β (fold)
- TNFα (fold)
- 18S
Figure 2.8 PepG- and CpG DNA-induced pro-inflammatory cytokine production in macrophages is blocked by triptolide. RAW264.7 cells were pretreated with indicated doses of triptolide for 30 min followed by stimulation with 10 μg/ml PepG (A) or 3 μg/ml CpG (B) for 6 h. The negative control was only treated with equal volume of vehicle (DMSO). Positive controls were those first treated with vehicle (DMSO) then stimulated with PepG. Upper panels represent the effect of triptolide on TNF-α and IL-6 secretion, which were assayed by ELISA. Results in the graph represent % relative to the mean value of the positive controls. Data were expressed as means ± S.E. from 3 independent experiments. *, p<0.05, compared to the group stimulated with PepG or CpG DNA after pretreatment with vehicle. Lower panels demonstrate the effect of triptolide on IL-1β production detected by Western blot analysis. Presented are the representative results of at least three experiments.
**Figure 2.9** The production of pro-inflammatory cytokines in PepG- and CpG-stimulated macrophages is strongly inhibited by triptolide. Primary peritoneal macrophages were pretreated with indicated doses of triptolide for 30 min followed by stimulation with 10 μg/ml PepG (A) or 3 μg/ml CpG DNA (B) for 6 h. The negative control was only treated with equal volume of vehicle (DMSO). Positive controls were those first treated with vehicle (DMSO) then stimulated with CpG. Upper panels represent the effect of triptolide on TNF-α and IL-6 secretion, which were assayed by ELISA. Results in the graph represent % relative to the mean value of the positive controls. Data were expressed as means ± S.E. from 3 independent experiments. *, $p<0.05$, compared to the group stimulated with PepG or CpG DNA after pretreatment with vehicle. Lower panels demonstrate the effect of triptolide on IL-1β production detected by Western blot analysis. Presented are the representative results of at least three experiments.
Figure 2.9

A

B
Figure 2.10  Triptolide inhibits the induction of miR-155 in LPS-stimulated macrophages. RAW264.7 cells were pretreated with the indicated doses of triptolide followed by stimulation with LPS for 4 h. Total RNA was harvested and Northern blot analysis was performed using a urea-PAGE denaturing gel. U6 was used as a loading control. Graph depicts the fold of changes in miR-155 expression in a representative experiment.
Figure 2.11 Triptolide attenuates LPS-induced cytokine production \textit{in vivo} in a dose-dependent manner. C57BL6 mice (5-6 weeks old) were given the indicated doses of triptolide, or equal volumes of vehicle i.p, and then challenged with LPS (5 mg/kg body weight). Cytokine concentrations in the serum were determined by ELISA. (A) Effects of triptolide on TNF-\(\alpha\) biosynthesis \textit{in vivo}. (B) Effects of triptolide on IL-6 production \textit{in vivo}. Values represent the mean \(\pm\) S.E. from 8-12 animals. †, \(p < 0.05\), compared to control group (not stimulated with LPS). *, \(p<0.05\), compared to the group received vehicle and then stimulated with LPS.
Figure 2.11

A

![Graph showing TNF-α levels](image)

B

![Graph showing IL-6 levels](image)
**Figure 2.12** Triptolide has no effect on IκBα degradation and p65 nuclear translocation, and does not affect NF-κB DNA-binding activity in LPS-stimulated macrophages. (A) Effects of triptolide on the components of the NF-κB pathway in LPS-stimulated macrophages. RAW264.7 cells were pretreated with 0.1 μM triptolide for 30 minutes, then stimulated with 100 ng/ml LPS for the indicated periods. Cells lysates were harvested and Western blotting was performed. Data shown were from a representative experiment. (B) Effect of triptolide on nuclear translocation of NF-κB p65 subunit. RAW264.7 cells were treated as in A, and immunofluorescence was performed with a p65 antibody (green), and then stained with DAPI (blue). The percentage of cells where p65 was localized in the nuclei was scored from at least 10 fields chosen randomly. Values in the graph on the right side represent mean ± S.E. from 3 independent experiments. (C) Effect of triptolide on NF-κB DNA-binding activity. RAW264.7 cells were treated as in A, and nuclear proteins were extracted. EMSA was performed using end-labeled double-stranded oligonucleotides containing a consensus NF-κB-binding element. Results were from a representative experiment.
Figure 2.12

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- IKK
- p-IκBα
- IκBα
- p50
- p65
- β-actin

B

Control

LPS

LPS + Triptolide

merge

C

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- NF-κB

Graph

Cells with p65 in nuclei (%)

Time (min)

- LPS
- LPS + Triptolide
Figure 2.13 Triptolide blocks gene transcriptional induction regulated by a variety of transcriptional factors. Effect of triptolide on reporter expression mediated by the transactivation domains of p65 NF-κB (A) and VP16 (B). RAW264.7 (A) and CHO-AA8-Luc (B) cells were treated as described. Cells were then lysed and luciferase activity in the lysates was measured. Relative luciferase units were normalized to protein concentrations. Luciferase activities in both A and B were expressed in the graphs as fold change relative to cells kept in tetracycline-containing medium (controls). Values represent mean ± SE from 3 independent experiments. † different from control group (with tetracycline), p<0.05. *, different from the group without tetracycline but treated with vehicle (DMSO), p<0.05. (C) Effect of triptolide on luciferase reporter mRNA expression mediated by the VP16 transactivation domain. CHO-AA8-Luc cells were treated as in B. Cells were harvested to isolate total RNA, and Northern blot analysis was performed. Luciferase expression was normalized to 18S rRNA, and expressed as fold increase relative to control (signal of cells grown in medium containing only tetracycline). (D) Triptolide inhibits the induction of Hsp70 by heat shock. RAW264.7 cells were subjected to heat shock in the presence of indicated doses of triptolide, and Northern blot analysis was performed. (E) Triptolide does not inhibit gene transcription by RNA polymerase II in vitro. RAW264.7 cells were either left untreated (C) or treated with vehicle (V) or triptolide (T), and in vitro transcription assays were performed using the indicated templates.
Figure 2.13

A

Relative RLU (fold)

Triptolide - - 0.1 (µM)
Tetracycline + - -

B

Relative RLU (fold X 10^4)

Triptolide - 0.1 - 0.05 0.1 (µM)
Tetracycline + + - -

C

Triptolide
Tetracycline
Heat shock

- 0.05 0.1 - 0.01 0.02 0.05 0.1 0.2 0.5 1 (µM)

- luciferase
(fold)

- 18S

D

Triptolide
Heat shock

- - 0.01 0.02 0.05 0.1 0.2 0.5 1 (µM)

- Hsp70
- 18S

E

Activated
Basal

C V T C V T

pG5HM
pMLΔ53
Figure 2.14 The hydroxyl group of triptolide plays a pivotal role in its anti-inflammatory action. RAW264.7 macrophages were pretreated with indicated concentrations of triptolide or TD-101 for 30 min, and then stimulated with LPS (100 ng/ml) or vehicle (PBS) for 6 h. The positive control was pretreated with equal volume of DMSO before LPS stimulation. The negative control was also pretreated with DMSO but not stimulated with LPS. For assaying the effects on TNF-α, $10^5$ cells were plated in each well. For assaying the effects on IL-1β, $3\times10^6$ cells were seeded in each 100-mm plate. (A) Comparison of the inhibitory effects of triptolide on TNF-α production with those of TD-101. TNF-α concentrations in the media were assayed by ELISA. (B) The effects of triptolide and TD-101 on LPS-stimulated IL-1β production. Cells were harvested and IL-1β levels in the lysates were detected by Western blot analysis using a monoclonal antibody specific for murine IL-1β. The same membranes were stripped and reprobed with a monoclonal antibody against β-actin to verify comparable protein loading.
Figure 2.14

A

![Bar graph showing TNF-α levels in response to Triptolide or TD-101 dose](image)

- **C** vs. **+ LPS**
- Triptolide: black bars
- TD101: white bars

B

![Western blot analysis](image)

- LPS: -, +, +, +, +, +, +, +
- Triptolide: +, +, +, +, +, +, +, +
- TD101: +, +, +, +, +, +, +, +
- IL-1β
- β-actin
Figure 2.15  The hydroxyl group of triptolide is essential for its apoptosis-promoting activity. Comparison of triptolide and TD-101 in their apoptosis-promoting activities. HeLa cells were either treated with increasing concentrations of triptolide or TD-101 for 24 h. Apoptotic cells were determined based on cellular morphology.
CHAPTER 3

ROLE OF MKP-1 IN CONTROL OF INFLAMMATORY RESPONSES

3.1 Introduction

Cytokine biosynthesis in phagocytes, including macrophages, relies on a series of signal transduction cascades initiated by microbial components through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) (149). For example, recognition of lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria, by TLR4 triggers a cascade of signaling events that lead to activation of transcription factor NF-κB and the MAP kinase pathways. The MAP kinase family plays a crucial role in mediating the induction of a number of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, through multiple mechanisms involving both transcriptional and post-transcriptional regulatory events. In addition to regulating gene transcription, MAP kinases can also modulate protein expression by altering the stability, transport, and translation of mRNA species that contain AU-rich elements (AREs) (51).

Messenger RNA turnover is an important process involved in the transient response to microbial insults and environmental stimuli. ARE consensus sequences (e.g. AUUUA and UUAUUUAUU) are present in the 3' untranslated region (UTR) of selected
mRNAs including interferons, cytokines (e.g. TNF-α), and proto-oncogenes (e.g. c-fos and k-ras) (150, 151). ARE-containing mRNAs encode proteins that are critical for many biological processes including cell growth and differentiation, signal transduction, transcriptional and translational control, hematopoiesis, apoptosis, nutrient transport, and metabolism (150). The balance between mRNA synthesis and decay determines the steady-state level of each species of mRNA. ARE-containing mRNAs are generally short-lived and they are rapidly degraded once their transcription terminates. Inadequate stabilization of ARE-containing mRNAs can cause a prolonged biological response that may subsequently lead to the development of disease (150). Abnormal stabilization of transcripts encoding growth-regulatory proteins such as c-myc and c-fos is associated with the development of malignancy (152-154). Stress activated protein kinases such as p38 MAP kinase plays an important role in stabilization of certain ARE-containing mRNAs such as TNF-α, IL-1β, IL-8, COX-2, and VEGF (155-157).

Nitric oxide (NO) plays an important role in immune defense against microbial pathogens (158). In response to bacterial infection, tissue resident macrophages are activated via pathways mediated by Toll-like receptors and serve as the first line of host defense by engulfment of bacterial pathogens through phagocytosis (2). Once the microbial pathogens are ingested, macrophages can produce a variety of toxic products, including NO, that kill the engulfed microorganisms. During microbial infection NO is primarily produced by the relatively high-output inducible NO synthase (iNOS) (2, 158). In addition to the microbicidal function, NO also acts as a potent vasodilator, facilitating the mobilization of antibodies, complement components, and effector leukocytes into the site of infection (158). While proper production of NO is crucial for host defense,
overzealous release of NO can disrupt the circulatory system, cause shock and ischemic injury to vital organs, leading to multi-organ failure (159). In the United States, septic shock accounts for approximately 250,000 deaths annually (160, 161).

Previously we have identified MAP kinase phosphatase (MKP)-1 as a crucial negative regulator of the inflammatory response during bacterial infection (97, 98, 100, 122, 162). We have shown that MKP-1 acts as a feedback control regulator for p38 and JNK MAP kinases: by dephosphorylating these MAP kinases the inducible MKP-1 protein terminates the p38 and JNK pathways, and switches off the inflammatory cascades. We examined the effect of MKP-1 on pro-inflammatory cytokine and iNOS mRNA stability. We found that Mkp-1-deficient macrophages expressed higher levels of pro-inflammatory cytokine mRNA as well as miR-155 than did wild-type cells. We also demonstrated that Mkp-1 deficiency stabilizes pro-inflammatory cytokine and iNOS mRNA transcripts. Our studies suggest that MKP-1 regulates pro-inflammatory cytokine and iNOS expression through post-transcriptional mRNA stabilization.

3.2 Materials and Methods

*Animals – Mkp-1+/+ and Mkp-1−/− mice were described previously (100, 122, 163). All animal experiments were carried out in accordance with the guidelines of the National Institutes of Health. The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Research Institute at Nationwide Children’s Hospital.*
Isolation of murine peritoneal macrophages and cell treatments – Thioglycollate-elicited peritoneal macrophages were isolated from mice by peritoneal lavage as previously described (122). The macrophages were cultured overnight in complete medium before being stimulated with 100 ng/ml LPS (Escherichia coli O55:B55; Calbiochem, La Jolla, CA). Actinomycin D (Calbiochem, La Jolla, CA) was added to the medium at a given time point to block mRNA synthesis, and mRNA decay was assessed by monitoring the change of the mRNA levels over time.

Northern blotting – Total RNA was isolated with TRIzol (Invitrogen, Carlsbad, CA). Northern blot analysis was carried out using murine IL-1β, TNF-α, IL-6, and iNOS cDNA probes, as previously described (97, 98). The membrane was stripped and reprobed with 18S rRNA or GAPDH cDNA to normalize for RNA loading. Quantitation of mRNA signals was performed with a Storm 860 system and ImageQuant TL software (Amersham Biosciences, Piscataway, NJ).

To examine the effect of Mkp-1 on miR-155 expression, total RNA were either isolated from stably transfected RAW264.7 cells that harbor an empty vector, a vector expressing Mkp-1 siRNA (Mkp-1 knockdown), or a vector expressing Mkp-1 cDNA (Mkp-1 over-expression) (97, 98). Total RNA (20 μg) was separated by electrophoresis on a 12% denaturing urea polyacrylamide gel. RNA was transferred onto Hybond N+ membrane (Amersham, Piscataway, NJ) using a Transblot cell (Bio-Rad, Hercules, CA). RNA was cross-linked to the membrane using UV irradiation. The antisense miR-155 and U6 probes were labeled with [α-32P]-dATP using StarFire Nucleic Acid Labeling System (IDT, Coralville, IA). The membrane was hybridized with a radioactive probe.
overnight at 37°C in a solution containing 7% SDS and 200 mM Na₂HPO₄ (pH 7.2). After washing 3 times at 37°C in a solution containing 2× SSPE and 0.1% SDS, the membrane was exposed to a phosphorimaging screen at room temperature for 1 h. The same membrane was stripped and rehybridized with a probe for the small nuclear RNA U6 to ensure comparable sample loading and for signal normalization.

*Pro-inflammatory cytokine and iNOS mRNA stability assays* – Thioglycollate-elicited peritoneal macrophages from *Mkp-1*⁺/⁺ and *Mkp-1*⁻/⁻ mice were stimulated with LPS (100 ng/ml) for a given time followed by treatment with the transcription blocker, actinomycin D (5 μg/ml), for different periods of time. Total RNA was isolated from the cells for Northern blot analysis. To examine the effect of p38 on iNOS mRNA stability in *Mkp-1* knockout cells, thioglycollate-elicited peritoneal macrophages from *Mkp-1*⁻/⁻ mice were stimulated with LPS (100 ng/ml) for 23 h followed by treatment with 10 μM SB203580 for 1 h. Then, the decay of cytokines and iNOS mRNA was analyzed.

### 3.3 Results

*The effect of Mkp-1 deficiency on pro-inflammatory cytokine mRNA induction in primary peritoneal macrophages* – It has been demonstrated that *Mkp-1* deficient cells produced higher levels of pro-inflammatory cytokine proteins than wild-type cells. Therefore we examined whether the lack of *Mkp-1* would also affect cytokine expression at the mRNA level. Macrophages from *Mkp-1* wild-type and knockout mice were treated with LPS for various times. Total RNA was harvested and levels of TNF-α and IL-1β mRNA were assessed by Northern blotting. Untreated control cells had undetectable levels of both
cytokines (Figure 3.1). Upon treatment with LPS, levels of TNF-α mRNA in wild-type cells increased after 30 min and peaked at 1 h, and remained fairly steady 6 h after stimulation with LPS (Figure 3.1A). In Mkp-1-deficient cells, TNF-α mRNA levels were elevated after 30 min and reached a peak at 2 h and then plummeted rapidly (Figure 3.1A). Expression of TNF-α mRNA in response to LPS was higher in Mkp-1-deficient than wild-type cells. Upon stimulation with LPS, we observed an increase in IL-1β mRNA levels in both wild-type and Mkp-1−/− cells after 1 h (Figure 3.1B). Levels of IL-1β mRNA increased steadily in both wild-type and Mkp-1−/− cells, peaked at 4 h after LPS treatment then started to decline (Figure 3.1B). Two, four and six h post challenge with LPS, expression of IL-1β mRNA was higher in Mkp-1-deficient than wild-type cells (Figure 3.1B).

_Messenger RNA of pro-inflammatory cytokines is more stable in LPS-stimulated Mkp-1-deficient macrophages than in wild-type cells –_ Since cells lacking Mkp-1 expressed higher levels of pro-inflammatory cytokine mRNAs than did Mkp-1 wild-type cells, we examined whether the stabilities of pro-inflammatory cytokine mRNAs were different between Mkp-1 wild-type and null cells. Peritoneal macrophages were treated with LPS for 2 h to induce the expression of different several cytokines. These cells were then exposed to actinomycin D to stop gene transcription and allow RNA to decay. Total RNA was isolated at various time-points, and the decay of pro-inflammatory cytokine mRNAs was assessed by Northern blotting. TNF-α mRNA underwent a more rapid decay in the wild-type macrophages than in the Mkp-1 knockout cells (Figure 3.2A). The
half-life of TNF-α mRNA in wild-type cells was approximately 30 min. In contrast, the half-life of TNF-α mRNA in the Mkp-1-deficient macrophages was around 45-90 min (Figure 3.2A).

Similarly, IL-1β mRNA decayed more rapidly in the wild-type macrophages than in the Mkp-1 knockout cells (Figure 3.2B). Two hours following LPS stimulation, IL-1β mRNA half-life in wild-type cells was approximately 30 min (Figure 3.2B). On the other hand, the levels of IL-1β mRNA in the Mkp-1-deficient macrophages remained steady throughout the 3 h treatment period and did not significantly decrease (Figure 3.2B).

Furthermore, IL-6 mRNA exhibited more rapid decay in wild-type macrophages compared to Mkp-1 knockout cells (Figure 3.2C). Two hours following LPS stimulation, the half-life of IL-6 mRNA in wild-type cells was approximately 30 min (Figure 3.2C). However, sustained levels of IL-6 mRNA were observed in Mkp-1-deficient macrophages (Figure 3.2C). Treatment with actinomycin D resulted in negligible IL-6 mRNA decay in knockout cells.

These results indicate that Mkp-1 deficiency enhances pro-inflammatory cytokine mRNA stability. Our results suggest that Mkp-1 regulates cytokine production in part by modulating the stability of cytokine transcripts through a process mediated by p38 and/or JNK.

*Messenger RNA of iNOS induced by LPS is more stable in Mkp-1-deficient macrophages than in wild-type cells* – It has been shown that iNOS mRNA stability can be regulated post-transcriptionally through a JNK-mediated pathway (164, 165). Several recent
studies, including one from our own laboratory, have demonstrated that *Mkp-1* deficiency leads to prolonged JNK and p38 activation (95, 100, 122, 166, 167). Thus, we examined whether the stabilities of iNOS mRNA were different between macrophages isolated from wild-type mice and those isolated from *Mkp-1* knockout mice. Peritoneal macrophages were treated with LPS for 24 h and then exposed to actinomycin D to stop gene transcription and allow RNA to decay. Total RNA was isolated at various time-points, and the iNOS mRNA decay was assessed by Northern blotting. As shown in Figure 3.3A, iNOS mRNA underwent a more rapid decay in the wild-type macrophages than in the *Mkp-1* knockout cells. Twenty-four h after LPS, the half-life of iNOS mRNA in wild-type cells was approximately 3.5 h. In contrast, the half-life of iNOS mRNA in the *Mkp-1*-deficient macrophages was estimated to be approximately 5.5 h (Figure 3.3A).

To answer the question whether p38 plays a role in the regulation of iNOS mRNA stability, we stimulated the *Mkp-1*+/− macrophages with LPS for 23 h followed by treatment with a pharmacological inhibitor of p38, SB203580, for 1 h and then examined iNOS mRNA decay in the presence of the p38 inhibitor (Figure 3.3B). The presence of p38 inhibitor decreased the half-life of iNOS mRNA from ~5.5 h to ~4 h. These results indicate that enhanced stability of iNOS mRNA in *Mkp-1*-deficient macrophages is at least partially due to elevated p38 activity.

*Mkp-1*−/− macrophages express higher level of miR-155 than do wild-type macrophages – Recent studies have demonstrated that microRNA plays a significant role in inflammatory responses (168, 169). Jing et al. have shown that miR-16 is involved in the regulation of the stability of cytokine mRNAs containing AREs (170). Moreover,
O’Connell et al. have demonstrated that miR-155 is induced in macrophages in response to both polyI:C and inflammatory cytokines (171). Furthermore, Tili et al. have provided evidence that miR-155 enhances the production of TNF-α in macrophages stimulated with LPS (172). Since Mkp-1-deficient macrophages exhibit dramatic changes in their response to inflammatory stimuli, we analyzed the microRNA expression profile using microRNA array. The microRNA, miR-155, was identified as one of the microRNAs whose expression was enhanced by Mkp-1 knockout. Northern blot analysis confirmed that miR-155 expression was enhanced in response to LPS stimulation. The effect of Mkp-1 on miR-155 expression was more clearly shown in RAW 264.7 macrophages expressing Mkp-1 cDNA or small interfering Mkp-1 RNA (siRNA). miR-155 expression was induced by LPS in RAW 264.7 harboring a control plasmid (Figure 3.4). Both the basal and LPS-induced miR-155 expression levels were attenuated by overexpression of Mkp-1 cDNA. Compared to RAW 264.7 cells carrying an empty vector, RAW 264.7 cells expressing an Mkp-1 siRNA produced markedly greater levels of miR-155.

3.4 Discussion
We have previously shown that in response to LPS challenge mice deficient in Mkp-1 more readily develop severe hypotension and shock than do Mkp-1-sufficient mice (122). To understand the molecular mechanism underlying the increased sensitivity of Mkp-1 knockout mice to endotoxin, we studied the role of Mkp-1 in the induction of a number of pro-inflammatory cytokines as well as iNOS. We have focused on iNOS and these cytokines since they play a pivotal role in the progress of septic shock and are involved in the pathogenesis of many inflammatory diseases. Highlighting the critical role of TNF-α
in the pathogenic process of endotoxemia, knockout of Tnf-α gene in mice and administration of TNF-α neutralizing antibodies prevent the mortality induced by LPS (173). In addition to inflammatory cytokines, excessive production of NO by iNOS is considered as a crucial event underlying vasodilatory shock and associated multi-organ failure. This study aims to address the mechanism for both augmented cytokine production and enhanced NO generation in Mkp-1-deficient mice.

Lack of Mkp-1 stabilizes pro-inflammatory cytokine mRNA transcripts – We and others have shown that LPS-induced TNF-α production by Mkp-1-deficient macrophages is substantially more robust than that by wild-type macrophages (122, 166, 167, 174). In this report, we found that Mkp-1 deficiency results in increased production of pro-inflammatory cytokines at the mRNA level (Figure 3.1). Additionally, we found that cytokine mRNAs (such as TNF-α, IL-1β, and IL-6) exhibited longer half-lives in Mkp-1-deficient macrophages (Figure 3.2). Another finding is that Mkp-1 deficiency differentially affects the half-lives of distinct cytokines. For example, absence of Mkp-1 almost completely prevented IL-1β and IL-6 decay. However, TNF-α decays was only modestly slowed down by Mkp-1 knockout (Figure 3.2). This is likely due to the fact that the mRNA of TNF-α is less stable overall, compared to IL-1β and IL-6. Many pro-inflammatory cytokine transcripts, including TNF-α, IL-1β, IL-6, granulocyte macrophage colony stimulating factor (GM-CSF), and IL-2, contain AREs in their 3′-untranslated regions and have been shown to be targets of tristetraprolin (TTP)-mediated mRNA decay (175). These mRNAs undergo stabilization mediated by MAP kinase-
regulated processes. It has been demonstrated that TTP binds to the AREs present in the 3′-untranslated regions of several cytokine mRNAs, and promotes the deadenylation and degradation of the mRNAs (176). The MAP kinase p38 can enhance the activity of MAP kinase-activated protein kinase (MK)-2 through phosphorylation (177). MK-2 in turn phosphorylates and inhibits the TTP-mediated degradation of ARE-containing mRNAs. The stabilization of cytokine mRNAs in Mkp-1 knockout cells can be explained by the less efficient deactivation of p38 and JNK in these cells. We have previously shown that while p38 and JNK activities in LPS-stimulated wild-type macrophages rapidly return to basal levels, the activities of p38 and JNK in Mkp-1-deficient macrophages last significantly longer. It is plausible that prolonged p38 activity in Mkp-1-deficient cells prevents TTP-mediated cytokine mRNA degradation and contributes to their enhanced stability. As a result, cytokine mRNAs are more stable in the Mkp-1-deficient cells than in the wild-type cells after LPS stimulation, as shown in the present study (Figure 3.2). It is likely that stabilization of cytokine mRNA transcripts in knockout macrophages contributes significantly to the higher cytokine levels in these cells.

Stabilization of iNOS mRNA in Mkp-1-deficient cells – Numerous reports have shown previously that stabilization of iNOS mRNA contributes to its induction in macrophages (165, 178-181). JNK, in particular, has been implicated in the stabilization of iNOS mRNA in macrophages (165). We found that iNOS mRNA stability was significantly increased in Mkp-1-deficient macrophages (Figure 3.3A). Furthermore, we found that pharmacological inhibition of p38 leads to a decrease in iNOS mRNA half-life in Mkp-1-deficient macrophages, suggesting that the increased iNOS mRNA stability is at least
partially due to the elevated p38 activity in these cells (Figure 3.3B). The mouse iNOS mRNAs contain a number of AUUUA elements in their 3'-UTR. Although JNK and HuR have been implicated in the stabilization of iNOS (165, 178), our study is the first to implicate Mkp-1 and p38 in the regulation of iNOS mRNA stability. This can be explained by the less effective inactivation of p38 and JNK in the Mkp-1⁻/⁻ cells. While p38 and JNK activities in LPS-stimulated wild-type macrophages return quickly to basal levels, the activities of p38 and JNK in Mkp-1-deficient macrophages last significantly longer. As the result, iNOS are more stable in the Mkp-1-deficient cells than in the wild-type cells after LPS stimulation, providing a reasonable explanation for the dramatic increase in iNOS in LPS-stimulated macrophages and hypotension in LPS challenged Mkp-1⁻/⁻ mice (Figure 3.3).

Expression of miR-155 is elevated in Mkp-1⁻/⁻ macrophages – We found that miR-155 expression was enhanced in Mkp-1-deficient or Mkp-1 knockdown macrophages (Figure 3.4). Previously, O'Connell et al. have shown that miR-155 is highly induced by TLR ligands, and its induction is mediated by JNK and involves TNF-α-mediated autocrine signaling (171). We have previously shown that JNK activity in LPS-stimulated macrophages is augmented in Mkp-1-deficient macrophages (122, 162). Because Mkp-1 is absent, these macrophages not only possess higher JNK activity due to the absence of the Mkp-1-mediated feedback control mechanism following the initial LPS stimulation, they are also subject to a stronger secondary stimulation by autocrine cytokines, such as TNF-α, through their respective receptors. Therefore, it is not surprising that miR-155 expression is significantly enhanced in the Mkp-1-deficient macrophages. Consistent
with this model, we found that the RAW 264.7 macrophages that over-express Mkp-1 exhibited a substantial decrease in miR-155 (Figure 3.4).

Our results have shown that Mkp-1-deficient cells produce higher levels of both miR-155 and pro-inflammatory cytokines. Mkp-1 deficiency also stabilizes cytokine mRNA transcripts. Since microRNAs have been shown to modulate gene expression by regulating the half-life of mRNAs or by inhibition of translation through binding to the 3'-UTR of target genes (182), we speculated that increased production of miR-155 can contribute to enhanced cytokine stability in cells lacking Mkp-1. Studies by Tili et al. have suggested that miR-155 increases TNF-α translation, possibly in part by enhancing TNF-α transcript stability (172). They demonstrated that linking the 3'-UTR of TNF-α mRNA to a GFP coding sequence increased the GFP reporter activity in a transient transfection assay with miR-155. Moreover, mice overexpressing miR-155 in B cells produced more TNF-α when challenged with LPS (172, 183, 184), highlighting the physiological significance of miR-155 expression on TNF-α production. Such, it is possible that over-expression of miR-155 in the Mkp-1-deficient macrophages and mice contributed to the excessive production of TNF-α and associated pathophysiology. Previously, Tili et al. have hypothesized that miR-155 may stabilize TNF-α mRNA by binding to its 3'-UTR (172). Although we cannot rule out such a possibility, we consider this unlikely. First, there is a consensus targeting site at the 3'-UTR of TNF-α. Second, directly targeting of mRNA by microRNA usually results in translational repression or degradation of the mRNA species. We think it is more likely that miR-155 may stabilize the TNF-α mRNA indirectly, possibly by attenuating the expression of a protein(s) that
mediates TNF-α mRNA degradation. Since IL-1β and IL-6 mRNAs also contain AREs in their 3'-UTRs, we speculated that miR-155 may also enhance IL-1β and IL-6 mRNA stabilities through a similar mechanism.

3.5 Figures

Figure 3.1 Pro-inflammatory cytokine mRNA levels are elevated in Mkp-1-deficient macrophages following LPS stimulation. Thioglycollate-elicited peritoneal macrophages were treated with LPS (100 ng/ml) for the indicated times. Total RNA was isolated and Northern blot analysis was used to determine TNF-α (A) and IL-1β (B) mRNA levels. Cytokine mRNA levels were normalized to 18S and the quantified data are presented graphically.
Figure 3.1

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Relative TNF-α mRNA (fold)

B

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Relative IL-1β mRNA (fold)
Figure 3.2 Mkp-1 deficiency stabilizes pro-inflammatory cytokine mRNA. Thioglycollate-elicited peritoneal macrophages were treated with LPS (100 ng/ml) for 2 h and actinomycin D (5 μg/ml) was then added to the medium to block transcription. Total RNA was isolated at indicated times after the addition of actinomycin D. Northern blot analysis was used to determine levels of TNF-α (A), IL-1β (B), and IL-6 (C) mRNA levels. Cytokine mRNA levels were normalized to 18S and the quantified data are presented graphically.
Figure 3.2

A  + LPS

\[ \text{Mkp-1}^{+/+} \quad \text{Mkp-1}^{-/-} \]

- TNF-\(\alpha\)

- 18S

B  + LPS

\[ \text{Mkp-1}^{+/+} \quad \text{Mkp-1}^{-/-} \]

- IL-1\(\beta\)

- 18S

C  + LPS

\[ \text{Mkp-1}^{+/+} \quad \text{Mkp-1}^{-/-} \]

- IL-6

- 18S

\[ \text{Mkp-1}^{+/+} \quad \text{Mkp-1}^{-/-} \]

% TNF-\(\alpha\) mRNA remaining

\[ \text{Time (h)} \]

\[ \text{Mkp-1}^{+/+} \quad \text{Mkp-1}^{-/-} \]

% IL-1\(\beta\) mRNA remaining

\[ \text{Time (h)} \]
**Figure 3.3** Knockout of *Mkp-1* enhances the stability of iNOS mRNA through a p38-mediated process. Thioglycollate-elicited peritoneal macrophages were first treated with LPS (100 ng/ml), and actinomycin D (5 μg/ml) was then added into the medium to block gene transcription. Total RNA was isolated at different times after addition of actinomycin D. Northern blot analysis was used to determine iNOS mRNA level. Levels of iNOS mRNA were normalized to 18S and the quantified data are presented graphically. (A) iNOS mRNA decay assay following 24 h of LPS stimulation. (B) Effects of p38 inhibitor on iNOS mRNA stability. *Mkp-1*-deficient peritoneal macrophages were first stimulated with LPS for 23 h, then treated with SB203580 (10 μM) for 1 h. Actinomycin D was added thereafter and iNOS mRNA decay was examined.
Figure 3.3

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Act D 0 0 1 2 3 4 8

-iNOS

-GAPDH

% mRNA remaining

Time (h)

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SB - - + + + + +

Act D 0 0 0 1 2 3 4 8

-iNOS

-GAPDH

% mRNA remaining

Time (h)
Figure 3.4 Effects of Mkp-1 over-expression or knock-down on miR-155 induction by LPS in RAW 264.7 macrophages. The establishment of RAW 264.7 cells harboring an empty vector, an Mkp-1 over-expression construct, or a construct expressing a Mkp-1 siRNA were previously described (97, 98). These stable clones were stimulated with LPS (100 ng/ml) or left untreated. miR-155 expression was examined by Northern blot analysis.
4.1 Introduction

Inflammatory bowel disease (IBD) commonly refers to ulcerative colitis (UC) and Crohn’s disease (CD), which are idiopathic, remitting and relapsing, chronic inflammatory diseases of the gastrointestinal tract (185-188). UC and CD differ in their location, pattern of distribution, depth of involvement within the intestinal mucosa, and histological lesions. IBD is often associated with extra-intestinal manifestations, affecting bones, joints, skin, eyes, hepatobiliary system, lungs, and kidneys (189, 190). Numerous studies have suggested that IBD is caused by inappropriate activation of mucosal immunity towards intestinal microflora, and/or dietary factors in individuals with a genetic predisposition (186, 188, 191). Traditionally, IBD is treated with anti-inflammatory drugs including 5-aminosalicylic acid compounds and corticosteroids as well as other immunomodulatory drugs to induce and maintain remission. In recent years, novel therapies targeting cytokines, receptors, and adhesion molecules have shown promising efficacy in treating IBD (41). In fact, because of the impressive efficacy, TNF-α neutralizing antibodies have been approved by FDA for the treatment of CD (192-194). More recently, neutralizing antibodies targeting TNF-α and IL-12 have
elicited favorable clinical responses in the treatment of UC and CD, respectively, highlighting the pivotal roles of these cytokines in the pathogenesis of IBD (40, 41, 195).

Interleukin-10 (IL-10) is one of the most potent anti-inflammatory cytokines that plays an important role in modulating the immune response (28). IL-10 is expressed in a variety of cell types, including T cells, macrophages, monocytes, dendritic cells, mast cells, B cells, eosinophils, keratinocytes, and epithelial cells (196). Numerous reports have demonstrated that the primary biological functions of IL-10 is limiting and terminating the inflammatory responses (196, 197). IL-10 can block the secretion of a large number of pro-inflammatory cytokines. Moreover, IL-10 also regulates the differentiation and proliferation of several sets of immune cells such as T, B, natural killer, and mast cells (198). Mice deficient in IL-10 exhibit augmented inflammatory responses, and develop endotoxic shock when challenged with LPS (29). When housed in a conventional environment, IL-10 knockout (KO) mice spontaneously develop enterocolitis between 7 and 11 weeks of age (199). In addition to colitis, IL-10 KO mice also exhibit anemia, weight loss, and increased mortality (199). The wasting syndrome and enterocolitis are associated with a substantial increase in the production of Th-1 cytokines, including TNF-α, IL-12, and IFN-γ (199-201). Adoptive transfer of CD4+ T cells from Il-10−/− mice to Rag-deficient mice induces chronic enterocolitis (202, 203), strongly supporting the idea that the pathogenesis in this animal model is mediated by colitogenic CD4+ T cells. The facts that anti-IL-12 and anti-IFN-γ monoclonal antibodies prevented colitis in the Il-10 KO mice, strongly suggest that enterocolitis in this model is predominantly a Th-1 disease (200). Highlighting the dependence of colitis in the Il-10
KO mice on gut flora, the severity of disease is greatly reduced when the $\text{Il-10}^{-/-}$ mice were housed in a specific pathogen-free (SPF) environment.

MAP kinase phosphatase (MKP)-1 is a dual specificity protein phosphatase preferentially inactivating p38 and JNK MAP kinases (63-68). Several laboratories, including ours, have demonstrated that MKP-1 plays a critical role in the regulation of the innate immune response, and $\text{Mkp-1}$-deficient mice are hyper-sensitive to TLR ligands and intact bacteria (122, 162, 166, 174). In this report, we examined the role of MKP-1 in the context of an $\text{Il-10}$ KO mouse model of IBD. We have found that mice lacking both $\text{Mkp-1}$ and $\text{Il-10}$ are highly susceptible to development of IBD characterized by inflammation and hyperplasia the intestinal tract, particularly the colon and rectum, even in SPF environment. Moreover, in addition to colitis, these double knockout mice also developed conjunctivitis and blepharitis, a manifestation of IBD in the ocular system. We have also shown that innate and adaptive immune effector cells of double KO mice are skewed towards an exaggerated $\text{T}_{h}-1$ response with excessive production of various pro-inflammatory cytokines. Our findings support that MKP-1 plays an important role in the prevention of IBD, and may have important clinical implications in the field of IBD.

4.2 Materials and Methods

$\text{Animals}$ – $\text{Mkp-1}$ knockout mice ($\text{Mkp-1}^{+/+}$ and $\text{Mkp-1}^{-/-}$) were provided by Bristol-Myers Squibb Pharmaceutical Research Institute (163). The $\text{Mkp-1}$ KO mice were back-crossed to 129 mice for at least 10 generations. $\text{Il-10}^{-/-}$ mice on a 129 genetic background were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were hosted in
an SPF facility. The \( Mkp-1^{-/-} \) mice on the 129 genetic background were crossed with the \( II-10^{-/-} \) mice to yield wild-type (\( Mkp-1^{+/+}/II-10^{+/+} \), \( Mkp-1^{-/-}/II-10^{+/+} \), \( Mkp-1^{+/+}/II-10^{-/-} \), and \( Mkp-1^{-/-}/II-10^{-/-} \) mice. Animals received humane care and all animal-related experiments were approved by the Institutional Animal Care and Use Committee of the Research Institute at Nationwide Children’s Hospital.

**Genotyping** – Mice were genotyped by PCR using genomic DNA isolated from tails as templates. Wild-type and mutant \( Mkp-1 \) alleles were detected in separate reactions. Two primers (5’-ATG GTG ATG GAG GTG GG C ATC CTG-3’ and 5’-CTG GTA GTG ACC CTC AAA GTG G-3’) were used to amplify the wild-type allele of \( Mkp-1 \). The knockout allele of \( Mkp-1 \) was amplified using primers 5’-CCA GGT ACT GTG TCG GTG GTG C-3’ and 5’-AGG TGA GAT GAC AGG AGA TC-3’. The wild-type and knockout alleles of \( II-10 \) were detected in a single reaction using three primers (5’-GTG GGT GCA GTT ATT GTC TTC CCG-3’, 5’-GCC TTC AGT ATA AAA GGG GGA CC-3’, and 5’- CCT GCG TGC AAT CCA TCT TG-3’), as recommended by The Jackson Laboratory.

**Monitoring mouse body weights and clinical scoring of IBD** – Body weights of \( Mkp-1^{+/+}/II-10^{+/+} \), \( Mkp-1^{-/-}/II-10^{+/+} \), \( Mkp-1^{+/+}/II-10^{-/-} \), \( Mkp-1^{-/-}/II-10^{-/-} \), and \( Mkp-1^{-/-}/II-10^{-/-} \) mice were monitored and recorded weekly starting at 3 weeks till they reached six months of age. The clinical scoring criteria were defined based on six parameters: loss of more than 5% of body weight; development of rectal prolapse; presence of perianal mucus; rectal bleeding; stool inconsistency; and death. Mice were given a score of either
0 (parameter absent) or 1 (parameter present) for each of those parameters, and the maximum possible score per mouse = 6. Scores were averaged from animals in each group (n > 10 for each group), and presented as mean ± S.E.

**Histology** – Large intestines were removed at necropsy from Mkp-1+/+/Il-10+/+, Mkp-1−/−/Il-10+/+, Mkp-1+++/Il-10−/−, and Mkp-1−/−/Il-10−/− mice. The tissue was flushed with cold 1× PBS and dissected into ascending, transverse, and descending colons as well as rectum. These tissue specimens were fixed in 10% formalin overnight and then placed in 1× PBS for 1 day. Eyes and eyelids were removed at necropsy from Mkp-1+/+/Il-10+/+, Mkp-1−/−/Il-10+/+, Mkp-1+++/Il-10−/−, and Mkp-1−/−/Il-10−/− mice. The specimens were fixed in 10% formalin for 4 days and then placed in 1× PBS for 1 day. Both intestinal and eye tissues were then embedded in paraffin, and cut into and 4-μm sections. The sections were stained with hematoxylin and eosin for histological evaluation.

**Histological scoring of inflammation and IBD in large intestine sections** – To calculate the inflammation score, H&E stained sections were blindly scored based on four criteria: inflammation severity, inflammation extent, crypt damage, and percentage of involvement (Table 4.1). The total inflammation score was calculated by adding the inflammation scores from the ascending, transverse, descending colons, and rectum. Therefore, the maximum possible inflammation score per animal is 56. The IBD scoring system was based on three parameters: mucosal hyperplasia, goblet cell depletion, and lymphoid aggregate hyperplasia (Table 4.2). Similarly, the scores of all 4 segments were added together to constitute the total IBD score. The maximum IBD score per animal is
Scores of all animals were averaged in each group (n= 3-4 per group), and presented as mean ± S.E.

Isolation of splenocytes, and lymphocytes – Spleens, axillary, cervical, inguinal and mesenteric lymph nodes were isolated from the mice, and single cell suspensions were prepared. The cells were collected, suspended in ACK buffer, and allowed to incubate for 3 min to lyse the red blood cells. After centrifugation, these cells were washed once with complete medium and finally cultured in RPMI 1640 medium (Mediatech) containing 10% FBS (Hyclone Laboratories).

CD4⁺ T cells were isolated from mouse lymph nodes by negative selection using the Miltenyi MACS® Purification kit (Auburn, CA) according to the manufacturer’s recommendations. Briefly, CD4⁺ T cells were enriched by depletion of cytotoxic T cells, B cells, NK cells, dendritic cells, macrophages, granulocytes and erythroid cells. Non CD4⁺ T cells were indirectly magnetically labeled by using a cocktail of biotin-conjugated antibodies against CD8a (Ly-2), CD45R (B220), DX5, CD11b (Mac-1), Ter-119, and Anti-Biotin MicroBeads. Isolation of highly pure CD4⁺ T cells was achieved via depletion of magnetically labeled cells by retaining them on a MACS® column in the magnetic field of a MACS® Separator, while the unlabeled CD4⁺ T cells pass through the column.

ELISA – Splenocytes and lymphocytes were plated into 6-well plates at a density of 2×10⁶ cells per well, and then stimulated with 100 ng/ml LPS (Escherichia coli 055:B5), purchased from Calbiochem (La Jolla, CA), for 24 h. IFN-γ, IL-12 (p70), TNF-α, and...
IL-6 concentrations in the media were assayed by ELISA, using commercial kits, as previously described (98, 100, 122). Intestines were harvested from 8 week old mice and divided into 5 parts: duodenum, jejunum, ileum, proximal colon and distal colon. These tissues were homogenized in lysis buffer and protein concentration was measured. IFN-\(\gamma\), IL-12 (p70), TNF-\(\alpha\), IL-6, IL-23 and IL-17A levels in the tissue homogenates were assayed by ELISA, and normalized to the corresponding protein concentrations.

CD4\(^+\) T cells were plated into 24-well plates at a density of \(1 \times 10^6\) cells per well, cells were then stimulated with plate-bound anti-CD3 (10 \(\mu\)g/ml) and anti-CD28 (10 \(\mu\)g/ml). IFN-\(\gamma\), IL-2, IL-4, IL-17A, and IL-5 concentrations in the media were assayed by ELISA as previously described (98, 100, 122).

**Northern blotting and Real-time RT-PCR** – Splenocytes and lymphocytes were treated with LPS for 24 h, and total RNA was collected using Trizol (Invitrogen, San Diego, CA). Northern blot analysis was carried out using mouse IL-12 p35 cDNA probe as described previously (97, 124). The membrane was stripped and reprobed with GAPDH to normalize for RNA loading.

To perform real-time PCR, the first-strand cDNA was synthesized using a reverse transcription kit (Invitrogen). Quantitative real-time PCR was performed using an ABI Prism 7900-HT sequence system (Applied Biosystems) with the QuantiTect SYBR Green PCR kit (Qiagen) in accordance with the manufacturer’s instructions. The following primers were used: IL12 (p40) forward: 5'-TTG CTG GTG TCT CCA CTC AT-3', and IL12 (p40) reverse: 5'-GGG AGT CCA GTC CAC CTC TA-3'; and hypoxanthine-guanine phosphoribosyltransferase (Hprt) forward: 5'-AGC CTA AGA TGA GCG CAA
GT-3', and Hprt reverse: 5'-TTA CTA GGC AGA TGG CCA CA -3'. The Hprt transcript was amplified as an internal control. The PCR reactions were performed in triplicate using 1μl of first-strand cDNA product, platinum Taq polymerase (Invitrogen), and gene-specific primer pairs. The threshold value of the ΔRn was set at 0.5. The PCR cycle where a significant increase in the ΔRn was first detected is referred to as the threshold cycle (C{T}). Relative expression was determined using the formula Rel Exp=2^{(ΔΔCT)} where ΔΔC_{T}=(C_{T} \text{ gene of interest}-C_{T} \text{ Hprt})_{\text{experimental sample}}-(C_{T} \text{ gene of interest}-C_{T} \text{ Hprt})_{\text{control sample}}.

Western blot analysis – Western blot analysis was carried out as previously described (97, 98, 100, 122). Rabbit polyclonal antibodies against phospho-p38, phospho-JNK and phospho-ERK were purchased from Cell Signaling (Danvers, MA). The blots were stripped, and probed with a β-actin monoclonal antibody (Sigma, St. Louis, MO), for verifying comparable sample loading.

Immunohistochemistry – Paraffin-embedded tissue sections of descending colons and rectum of Mkp-1^{+/+}/Il-10^{−/−} and Mkp-1^{−/−}/Il-10^{−/−} mice were deparaffinized in xylene and rehydrated in ethanol. Antigen retrieval was performed using a 0.01 M citrate buffer at pH 6.0 or 1 × EDTA pH 8 solution in a steamer for 30 min. The slides were then processed using a mouse to mouse HRP AEC staining kit from ScyTek (Logan, UT) according to the manufacturer’s recommendations. Briefly, sections were incubated overnight with antibodies against phospho-histone H3 (Upstate Biotechnology), CD3 (Sigma), B220 (University of California San Francisco, Hybridoma and Monoclonal
Antibody Core Facility), claudin-2 (Invitrogen), occludin (Invitrogen), and ZO-1 (Invitrogen). After the reactions with the peroxidase-labeled secondary antibodies, color was developed with 3-amino-9-ethylcarbazole (AEC), and the sections were counterstained with hematoxylin. A positive reaction was indicated by a red color.

Crypt length – Sections were scored as previously described (204). Average crypt length was calculated for Mkp-1<sup>+/+</sup>/Il-10<sup>−/−</sup> and double knockout mice as follows (3 per group). Twenty intact crypts were identified from both the colon and rectum, and the length of each crypt was measured from the crypt base basement membrane to the tip of the luminal epithelium using Image-Pro Plus (Media Cybernetics, Bethesda, MD). The data were expressed as the average crypt length in a given animal. The scores of the individual animals in a group were averaged and the data presented as mean ± S.E.

Statistics – The data obtained from the experiments were analyzed by one-way ANOVA with LSD post hoc test, using SPSS statistical software program (SPSS, Chicago, IL). Differences were considered significant when p < 0.05.

4.3. Results

Knockout of Mkp-1 on an Il-10-null background substantially predisposes mice to colitis and ocular inflammation – To assess the role of MKP-1 in the IL-10 knockout mouse model of IBD, we backcrossed MKP-1 knockout mice to 129 mice for more than 10 generations. These mice were then allowed to interbreed with IL-10 knockout mice, which also had a pure 129 background. By breeding these mice, we generated mice that
were wild-type (Mkp-1\(^{+/+}\)/Il-10\(^{++/+}\)), MKP-1 knockout (Mkp-1\(^{-/-}\)/Il-10\(^{++/+}\)), IL-10 knockout (Mkp-1\(^{+/+}\)/Il-10\(^{-/-}\)), or double knockout (Mkp-1\(^{-/-}\)/Il-10\(^{-/-}\)) mice. These mice were kept in an SPF environment. As expected, Mkp-1 knockout mice were normal and fertile, and no abnormality was observed in the absence of challenge. In our SPF facility, IL-10 knockout also appeared to develop and age normally, although rarely a small percentage of mice developed rectal prolapse. In contrast, the mice deficient in both Mkp-1 and Il-10 genes developed severe prolapse within 3-4 months after birth (Figure 4.1). Generally, these mice exhibited poor growth and high mortality. In fact, the prolapse was often so severe that for humane reasons these mice had to be euthanized shortly after the onset of the disease. In addition to this phenotype, double knockout mice also exhibited high prevalence of unilateral and bilateral eyelid edema (Figure 4.2A), indicated by signs of swelling of the upper and lower eyelids. Swelling was moderate to severe and routinely hindered visualization of the globe. No ocular discharge was observed. Thirty percent of double knockout mice developed both rectal prolapse and ocular disease, 20% developed only prolapse, while 10% developed only ocular disease, suggesting a potential mechanistic association between prolapse and ocular inflammation.

It has been demonstrated that mice lacking IL-10 develop chronic colitis indicated by growth retardation, prolapse, perianal mucus, rectal bleeding, and diarrhea (200, 205). We examined the effect of Mkp-1 deficiency on the growth and other signs of colitis. Mkp-1\(^{+/+}\)/Il-10\(^{++/+}\), Mkp-1\(^{-/-}\)/Il-10\(^{++/+}\), Mkp-1\(^{+/+}\)/Il-10\(^{-/-}\), Mkp-1\(^{-/-}\)/Il-10\(^{-/-}\), and Mkp-1\(^{-/-}\)/Il-10\(^{-/-}\) mice were monitored continuously from 3 weeks of age until reaching 6 months of age. The severity of clinical colitis was evaluated over this period. Mkp-1\(^{+/+}\)/Il-10\(^{++/+}\) and Mkp-1\(^{-/-}\)/Il-10\(^{++/+}\) mice did not show any signs of colitis. Colitis developed late in a few
"Mkp-1\(^{+/+}\)/Il-10\(^{-/-}\) mice (2 out of 13), and disease signs were generally mild. Double knockout mice showed higher levels of wasting. A higher portion of the double knockout mice (14 of 20) developed severe rectal prolapse often accompanied by both perianal mucus and rectal bleeding, than Mkp-1\(^{+/+}\)/Il-10\(^{-/-}\) mice. Surprisingly, loss of one copy of the Mkp-1 gene significantly accelerated the development of colitis (12 out of 23), suggesting that MKP-1 suppresses colitis development in a dose-dependent manner. Colitis almost always led to mortality within a few weeks in the double knockout mice. Overall, the clinical IBD score was 0 in both wild-type and Mkp-1\(^{+/+}\)/Il-10\(^{-/-}\) mice. Mkp-1\(^{+/+}\)/Il-10\(^{-/-}\) occasionally developed appreciable IBD signs, the scores were always mild within the 6-month observational period. In contrast, double knockout mice developed substantially more severe IBD, indicated by substantially higher clinical scores (Figure 4.3A).

Double knockout mice exhibited significantly higher histological IBD scores than Mkp-1\(^{+/+}\)/Il-10\(^{-/-}\) mice – H&E stained slides from large intestines (ascending, transverse, descending colons, and rectum) of 8-week old mice were scored blindly for inflammation using the scoring criteria shown in Table 4.1. Wild-type and Mkp-1\(^{-/-}\)/Il-10\(^{+/+}\) mice had histologically normal colons. Consistent with previous reports (206), mild inflammation was observed in the Mkp-1\(^{+/+}\)/Il-10\(^{-/-}\) mice. On the other hand, double knockout mice had elevated inflammatory scores (Figure 4.3B) and inflammation extended into the mucosa and submucosa, with transmural inflammation in some of the mice (Figure 4.4A). Inflammatory cells included neutrophils (suppurative colitis), plasma cells, B220\(^{+}\) B- and CD3\(^{+}\) T-lymphocytes, with the majority being T-lymphocytes (Figure 4.4B).
Double knockout mice had inflammation throughout ~50% of the colonic lumen, compared to ~25% of the colon in $Mkp-1^{+/+}/Il-10^{-/-}$ mice. Furthermore, inflammatory lesions tended to be continuous and widespread in the double knockout mice, whereas lesions in the $Mkp-1^{+/+}/Il-10^{-/-}$ mice were mostly isolated and smaller.

Additionally, H&E sections were blindly scored for mucosal epithelial lesions using the scoring criteria shown in Table 4.2 and mucosal hyperplasia was assessed by measuring crypt lengths in descending colon and rectum. Wild-type and $Mkp-1^{-/-}/Il-10^{+/+}$ mice had no mucosal lesions. Double knockout mice had more severe mucosal epithelial proliferation and crypt damage than in $Mkp-1^{+/+}/Il-10^{-/-}$ mice (Figure 4.4A). Mucosal lesions were seen both in greater total colon surface and in a larger portion of the individual crypt (~2/3 of individual crypt length) in double knockout mice than in $Mkp-1^{+/+}/Il-10^{-/-}$ mice (~1/3 of individual crypt length was affected). Crypt abscesses were occasionally seen in the double knockout mice but absent in $Mkp-1^{+/+}/Il-10^{-/-}$ mice. Double knockout colonic crypts had fewer goblet cells than did those in $Mkp-1^{+/+}/Il-10^{-/-}$ mice. Moreover, rectal crypts in double knockout mice were significantly longer than those in $Mkp-1^{+/+}/Il-10^{-/-}$ mice (503.6 ± 78.7 vs. 160.1 ± 24.9 μm, $p<0.05$), explaining the increased incidence of severe prolapse. To assess crypt epithelial cell proliferation, sections of the rectum were immunohistochemically labeled for phospho-histone H3, a mitotic marker. The hyperplastic rectums from double knockout mice had many more positive cells in the base of the crypts than the $Mkp-1^{+/+}/Il-10^{-/-}$ mouse rectums (Figure 4.4C). Overall, the IBD scores of the double knockout mice were significantly higher than those of the $Mkp-1^{+/+}/Il-10^{-/-}$ mice (Figure 4.3C).
Disruption of the epithelial barrier has been implicated in IBD development and progression (207, 208). We examined whether intestines of double knockout mice exhibited altered tight junction protein distribution using immunohistochemistry (IHC). Claudin-2 and ZO-1 staining were comparable in descending colons of both double knockout and $Mkp-1^{+/+}/Il-10^{-/-}$ mice (data not shown). On the other hand, occludin staining at the crypt epithelial apical surface was significantly weaker or absent in descending colons of double knockout mice compared to those of $Mkp-1^{+/+}/Il-10^{-/-}$ mice (Figure 4.4D). These findings indicate that the epithelial barrier integrity is more severely disrupted in descending colons of double knockout mice than in those of $Mkp-1^{+/+}/Il-10^{-/-}$ mice. Our studies suggest a potential role for MKP-1 in tight junction regulation.

**Double knockout mice often develop conjunctivitis and blepharitis** – Sections of eyes and eyelids were prepared and stained with H&E to characterize the peri-ocular lesions seen clinically in double knockout mice. Wild-type mice had normal conjunctival mucosa (Figure 4.5A). Both $Mkp-1^{-/-}/Il-10^{+/+}$ and $Mkp-1^{+/+}/Il-10^{-/-}$ mice had mildly thickened conjunctival mucosa, moderately increased goblet cell numbers, and minimal amounts of mucus within the conjunctival space (Figures 4.5B and C). In contrast double knockout mice had markedly thickened conjunctival mucosa with occasional intraepithelial abscesses. Double knockout mice also had moderate goblet cell hyperplasia, resulting in large amounts of free mucus within the conjunctival space. The conjunctival stroma and mucosa were also mildly to moderately edematous and often infiltrated with a large number of neutrophils and fewer lymphocytes and plasma cells (Figure 4.5D).
In wild-type, *Mkp-1<sup>−/−</sup>/Il-10<sup>+/+</sup> and *Mkp-1<sup>+/+</sup>/Il-10<sup>−/−</sup> mice (Figures 4.6A-C), the eyelid haired skin was histologically normal. In contrast, double knockout mice had markedly thicker (acanthotic) eyelids with marked chronic inflammatory cell infiltration in the submucosa that was primarily lymphocytic and plasmacytic (Figure 4.6D).

*Deletion of Mkp-1 further skews the immune system of Il-10 knockout mice toward Th-1 responses* – TNF-α plays an important role in the pathogenesis of IBD, and knockout of IL-10 genes enhances the production of TNF-α in response to LPS challenge (209). Since we have previously found that deletion of the *Mkp-1* gene dramatically augments TNF-α and IL-6 production (122), we asked the question whether deletion of *Mkp-1* on an *Il-10* null background further exaggerates TNF-α and IL-6 production. Cells were isolated from the spleens and lymph nodes (combined axillary, cervical, inguinal, and mesenteric lymph nodes) from wild-type, *Mkp-1<sup>−/−</sup>/Il-10<sup>+/+</sup>, *Mkp-1<sup>+/+</sup>/Il-10<sup>−/−</sup>, and double knockout mice. To assess the innate immune response, these cells were stimulated with LPS, and cytokine production was measured by ELISA. As expected, more TNF-α and IL-6 were produced by both *Mkp-1<sup>−/−</sup>/Il-10<sup>+/+</sup> and *Mkp-1<sup>+/+</sup>/Il-10<sup>−/−</sup> splenocytes than by wild-type splenocytes (Figure 4.7A). Likewise, *Mkp-1<sup>−/−</sup>/Il-10<sup>+/+</sup> and *Mkp-1<sup>+/+</sup>/Il-10<sup>−/−</sup> lymph node cells also produced greater amounts of TNF-α and IL-6 than do wild-type cells (Figure 4.7B). Splenocytes and mixture lymph node cells isolated from the double knockout mice produced the greatest quantities of both TNF-α and IL-6 (Figure 4.7), indicating that MKP-1 and IL-10 function cooperatively in restraining the inflammatory response.
Since it has been shown that lack of IL-10 skews the immune response towards one that is predominantly Th-1 (206), we examined whether lack of Mkp-1 would cause a further shift towards a hyper Th-1 response. For this reason, we measured IL-12 and IFN-γ production in LPS-stimulated splenocytes and lymph node cells (Figure 4.8A). Splenocytes and lymph node cells from Mkp-1+/+Il-10−/− mice produced greater quantities of IL-12, and IFN-γ than did their wild-type counterparts. Importantly, double knockout cells produced the highest levels of these Th-1 cytokines (Figure 4.8A). Northern blot analysis confirmed that IL-12 p35 expression induced by LPS in Mkp-1+/+Il-10−/− splenocytes was substantially enhanced by Mkp-1 deletion (Figure 4.8B). qPCR revealed similar results in LPS-treated lymph node cells (Figure 4.8B). These results indicate that knockout of Mkp-1 on the Il-10 null background facilitates innate immune reactions favoring an exaggerated Th-1 response.

As we have demonstrated that Mkp-1 deficiency causes a shift in the innate immune system towards a Th-1 response, we further examined the role of MKP-1 on the adaptive immune system. CD4+ T cells were isolated from lymph nodes (combined axillary, cervical, inguinal, and mesenteric lymph nodes) of Mkp-1+/+Il-10−/− and double knockout mice, and the T cell receptor (TCR) was activated by TCR cross-linking through incubation with plate-bound α-CD3 and α-CD28 antibodies. Production of IL-2, IFN-γ (Th-1 cytokines), IL-4 and IL-5 (Th-2 cytokines), and IL-17A (Th-17 cytokine) by CD4+ T cells were measured by ELISA. Cells isolated from double knockout mice produced significantly higher levels of IFN-γ than did those from Mkp-1+/+Il-10−/− mice (Figure 4.9A). However, production of IL-2, IL-17A as well as the Th-2 cytokines, IL-4
and IL-5 was comparable in CD4+ T cells from double knockout and Mkp-1+/+/Il-10−/− mice (Figure 4.9A).

To assess the functionality of MKP-1 in T lymphocytes, we examined MAP kinase activation kinetics in CD4+ T cells after TCR cross-linking. Western blot analyses using antibodies against phosphorylated MAP kinases indicate that CD4+ T cells isolated from double knockout mice exhibited slightly higher p38, JNK and ERK activities than did cells isolated from Mkp-1+/+/Il-10−/− mice (Figure 4.9B). These findings indicate that MKP-1 is involved in MAP kinase regulation in T lymphocytes.

*Intestines in double knockout mice contain higher levels of inflammatory cytokines than those in Mkp-1+/+/Il-10−/− mice and exhibit enhanced MAP kinase activity* – To further assess the physiological relevance of alterations in immune response in vitro, we assayed levels of inflammatory cytokines in intestinal tissues. Intestines were harvested from 8 week old double knockout and Mkp-1+/+/Il-10−/− mice, and these tissues were divided into five segments (duodenum, jejunum, ileum, proximal, and distal colons). Tissues were homogenized in a lysis buffer to extract proteins. Cytokine levels in the tissue homogenates were measured by ELISA. Small intestines of both double knockout and Mkp-1+/+/Il-10−/− mice had low levels of cytokines, consistent with the fact that colitis in this model primarily affects the large intestine. The proximal and distal colons of double knockout mice had much higher levels of IFN-γ, IL-12 (p70), TNF-α, IL-6, IL-23, and IL-17A than did those in Mkp-1+/+/Il-10−/− mice (Figure 4.10A).

We also examined MAP kinase activities in intestinal tissues. Colons in double knockout mice exhibited higher levels of phospho-p38, phospho-ERK and phospho-JNK.
than did colons in $Mkp-1^{+/+}/Ii-10^{-/-}$ mice (Figure 4.10B). Since MAP kinases are important modulators of signaling pathways leading to cytokine production, cell proliferation, and cell death, these findings suggest that abnormal MAP kinase regulation may be implicated in the exacerbated inflammatory response and the pathophysiology of colitis in double knockout mice.

### 4.4 Discussion

This study is the first to examine the role of MKP-1 within the intestinal tract. We found that mice lacking $Mkp-1$ and $Ii-10$ are susceptible to the development of rectal prolapse (Figure 4.1). Double knockout mice developed substantially more severe IBD, indicated by higher clinical, and histological scores compared to $Mkp-1^{+/+}/Ii-10^{-/-}$ mice (Figure 4.3). Colons of double knockout mice had marked inflammatory cell infiltration, as well as mucosal hyperplasia associated with enhanced epithelial cell proliferation (Figure 4.4). Additionally, double knockout mice developed peri-ocular lesions (Figure 4.2), including conjunctivitis (Figure 4.5) and blepharitis (Figure 4.6). Lack of $Mkp-1$ on an $Ii-10$ deficient background further skews the innate (Figures 4.7 and 4.8) and adaptive (Figure 4.9) immune systems towards an exaggerated T$_{h}$-1 response. Furthermore, colons of double knockout mice produced elevated levels of inflammatory cytokines and MAP kinases compared to $Mkp-1^{+/+}/Ii-10^{-/-}$ mice (Figure 4.10).

In this study we also demonstrate a dosage effect for MKP-1, where loss of one copy of the $Mkp-1$ gene in $Mkp-1^{+/-}/Ii-10^{-/-}$ mice makes them significantly more susceptible to the development of rectal prolapse than $Mkp-1^{+/+}/Ii-10^{-/-}$ mice (Figure 4.3A). This finding indicates that the suppression of colitis in the $Ii-10$ knockout mice by
MKP-1 is not only dependent on the presence of a functional *Mkp-1* gene, but also requires the expression of the *Mkp-1* gene at a sufficient level. This is consistent with the biochemical mechanism of dephosphorylation of MAP kinases by MKP-1 protein. Loss of one copy of the *Mkp-1* gene in *Il-10* null mice is likely to result in decreased MKP-1 protein levels, less efficient dephosphorylation of the MAP kinases, ultimately leading to compromise in immune responses and predisposition to the development of colitis.

**Ocular abnormalities** – The development of conjunctivitis (Figure 4.5) and blepharitis (Figure 4.6) in the double knockout mice was an intriguing finding, since clinical extraintestinal manifestations have not been reported in any rodent IBD model, including IL-10 KO mice. In people, extraintestinal manifestations can occur in the eyes, liver, skin, joints, kidneys, liver, biliary tracts, and vascular system but the mechanisms involved are not completely understood (189, 190). Extraintestinal IBD-related immune disease can be classified into two major groups: the first are often associated with intestinal inflammatory activity and likely share a similar pathogenesis with IBD; the second includes many autoimmune diseases that may have distinct mechanisms from bowel inflammation (190). Four to twelve percent of IBD patients show evidence of ocular disease including episcleritis, scleritis, uveitis, retinal vasculitis, and conjunctivitis (189, 190). While the double knockout mice develop conjunctivitis and blepharitis, inflammation in these mice did not appear to extend beyond peri-ocular tissues. This is an important distinction between IBD in human patients and double knockout mice. The exact mechanism for the development of ocular disease in this model is not known, but it might be secondary to the inflammation associated with colitis or a potential systemic increase in cytokines that affect the eye. Regardless of the mechanisms involved, our
double knockout mouse could be a valuable animal model for the development of therapy to treat the ocular manifestation of IBD.

Mechanistic insights regarding role of Mkp-1 in the development of IBD – The results of this study suggest several mechanisms by which loss of MKP-1 function may promote IBD-like disease in double knockout mice. Currently, IBD is believed to be a T-cell driven process, with inappropriate cytokine production by CD4⁺ T cells (210). Crohn's disease is associated with deregulated T₉-1 cytokine expression, whereas ulcerative colitis is associated with a T₉-2 response (210). Studies with the Il-10 KO mice have provided compelling evidence to support an exacerbated T₉-1 response as the underlying course of colitis. The studies presented here demonstrate that loss of MKP-1 enhances disease progression, in part by skewing both the innate and adaptive immune responses towards an exaggerated T₉-1 response (Figures 4.8 and 4.9). This notion is supported by enhanced production of T₉-1 cytokines by both the innate and the adaptive immune effector cells (Figures 4.8 and 4.9). Previously, we have shown that knockout of Mkp-1 enhances TNF-α, IL-6, and IL-10 production upon LPS stimulation in both macrophages and dendritic cells, but attenuated IL-12 and IFN-γ production (122). We have speculated in the previous report that over-production of IL-10 in the Mkp-1-deficient cells is likely responsible for the attenuated IL-12 and IFN-γ (122). Such speculation is supported by the findings presented here, which indicate markedly increased IL-12 and IFN-γ production in cells lacking both Mkp-1 and Il-10 genes after LPS stimulation. Moreover, CD4⁺ T cells in peripheral lymph nodes also produced more IFN-γ in response
to T cell receptor activation (Figure 4.9A). The enhanced T_{h-1} response observed in cultured cells is also reflected in vivo, since colonic tissues contain greater levels of T_{h-1} cytokines such as TNF-\(\alpha\), IFN-\(\gamma\), and IL-12 (Figure 4.10A).

While it is clear that peripheral CD4\(^+\) T cells exhibit an excessive T_{h-1} response, the mechanism underlying such alteration remains obscure. T helper cell differentiation and expansion is determined by cytokines present in the local environment. Since antigen-presenting cells deficient in both Mkp-1 and Il-10 produce increased T_{h-1} cytokines (Figure 4.8), it is easy to understand why greater T_{h-1} cell activity is present in the system. On the other hand, Mkp-1 deficiency in T lymphocytes alone may enhance MAP kinase activities in these cells and tilt the balance of the regulatory network toward a T_{h-1} response. Previously Lu et al. have shown that p38 is required in T_{h-1} cells for IFN-\(\gamma\) production (211). Similarly, JNK2 also plays an important role in the regulation of T_{h-1} cells, and deletion of JNK2 attenuates IFN-\(\gamma\) production in T cells (212). Thus it is also possible that deletion of Mkp-1 causes an intrinsic defect that favors the production of T_{h-1} cytokines. The finding that MAP kinase activity is appreciably altered as a result of Mkp-1 deletion supports this idea (Figures 4.9B and 4.10B).

In addition to elevated T_{h-1} cytokines, intestinal tissues of double knockout mice also contained higher levels of IL-17 and IL-23, raising the question of whether T_{h-17} cells are also involved in the intestinal and ocular manifestations of the double knockout mice. T_{h-17} differentiation depends on TGF-\(\beta\) and IL-6, and T_{h-17} cell expansion requires IL-23 (213). Although it is unclear whether TGF-\(\beta\) production in the mice was affected by Mkp-1 knockout, Mkp-1 deletion did enhance the production of IL-6 (Figure
The increases in the levels of both IL-6 and IL-23 in the double knockout mice suggest that the intestinal environment in the double knockout mice will likely favor the differentiation and expansion of Th-17 cells. It should be pointed out that Th-17 cells are not the only cell type capable of producing IL-17. In addition to Th-17 cells, stromal cells and myeloid-derived cells also produce IL-17 in an IL-23-dependent manner. Therefore, an increase in IL-17 in colonic tissues does not necessarily indicate the presence of Th-17 cells in the colonic tissues. Future experiments will address this issue.

Previously, it has been thought that IL-17 and Th-17 are culprits in the pathogenesis of colitis in the IL-10 knockout mice. IL-17 is known to regulate the production of chemokines by stromal cells and play a critical role in mediating immunity against extracellular bacteria. However, a recent paper by O’Connor et al. actually demonstrated a protective function for IL-17A in T cell-mediated intestinal inflammation (214). Other studies have also suggested that IL-17 might play a protective function in the gut in a T cell-independent model of wasting disease (215, 216). Thus, in our double knockout mice, the elevated levels of IL-17A in intestines of double knockout mice may represent a compensatory mechanism in an attempt to protect the intestinal mucosa from excessive inflammation, rather than a factor contributing to colonic inflammation. In this regard, our double knockout mice may offer an additional opportunity to study the role of Th-17 cells in an inflammatory disease model.

Cooperation between IL-10 and MKP-1 in regulating intestinal immune responses –

Our studies provided compelling evidence to support a model that by inactivating MAP kinases, MKP-1 cooperates with IL-10 to maintain the homeostasis and prevent the
development of IBD. In the colonic environment, tight junctions of the intestinal epithelium serve as a barrier to prevent the intestinal bacterial invasion into the submucosa. The barrier also prevents the exposure of immune effector cells in the submucosa to the highly inflammatory bacterial components in the intestine lumen. When the submucosa is exposed to residual amount of bacterial components due to minor epithelial damage or due to bacterial invasion, the immune effector cells in the submucosa initiate an inflammatory response through TLR. Consequently, MAP kinase activation occurs, leading to production of both pro-inflammatory and anti-inflammatory cytokines. The inflammatory cytokines are crucial for the elimination of invading bacteria and for the repair of barrier function through inducing epithelial cell proliferation. When both MKP-1 and IL-10 are present, the two molecules function together to reign in the inflammatory response and restore homeostasis in a cooperative manner. In this process, MKP-1 is crucial for terminating the MAP kinase signaling which drives the inflammation, whereas IL-10 is pivotal for pacification of downstream inflammatory actions. IL-10 energizes MKP-1-mediated feedback control of the inflammatory cascade through enhancing MKP-1 expression (95). In the absence of IL-10, the MKP-1-mediated feedback control becomes the predominant mechanism responsible for limiting the inflammatory response. This mechanism is sufficient to prevent excessive inflammatory response triggered by the intestinal microflora where intestinal bacteria are limited either in diversity or in quantity. This explains the absence of severe colitis in the Il-10 KO mice when they are housed in an SPF facility. However, in a conventional animal facility, in the absence of Il-10 the MKP-1-mediated feedback control mechanism may not be sufficient to protect the intestine from excessive
inflammation when pathogenic intestinal bacteria are present. When both MKP-1 and IL-10 are absent, even minor insults from normally harmless intestinal microflora present in an SPF facility may cause an overwhelming inflammatory response, leading to severe inflammation and damage in the gastrointestinal system. Since MKP-1 inhibits colitis severity in a dose-dependent manner, pharmacological enhancement of MKP-1 activity by increasing gene expression or protein stability may be beneficial for the treatment of IBD.

4.5 Figures and Tables

**Figure 4.1** Double knockout mice develop severe rectal prolapse. (A) Genotyping of *Mkp-1^{+/+}/Il-10^{+/+}, Mkp-1^{-/-}/Il-10^{+/+}, Mkp-1^{+/+}/Il-10^{-/-},* and *Mkp-1^{-/-}/Il-10^{-/-} mice* by PCR. (B) Only double knockout mice develop rectal prolapse. (C) Time course of rectal prolapse development. *p*<0.05, double knockout mice vs. other genotypes. (n=16 in each group).  

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Figure 4.1

A

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Δ II-10

-II-10

-Δ Mkp-1

-Mkp-1

B

Mkp-1⁻/⁻/II-10⁻/⁻

Mkp-1⁺⁺/⁻⁻/II-10⁻/⁻

Mkp-1⁻/⁻/II-10⁺⁺/⁺⁺

Mkp-1⁺⁺/⁻⁻/II-10⁺⁺/⁺⁺

C

% of mice with prolapse

Age (months)

0 1 2 3 4 5 6

Mkp-1⁻/⁻/II-10⁻/⁻

Mkp-1⁺⁺/⁻⁻/II-10⁻/⁻

Mkp-1⁺⁺/⁻⁻/II-10⁺⁺/⁺⁺
**Figure 4.2** Mice lacking both *Mkp-1* and *Il-10* genes develop high incidence of ocular disease. (A) Peri-ocular inflammation in double knockout mice. (B) Time course of peri-ocular inflammation. $p<0.05$, double knockout mice vs. other genotypes. (n=12 for each genotype).
Figure 4.2

A

Mkp-1⁻/⁻/Il-10⁻/⁻  Mkp-1⁺⁺/⁺⁺/Il-10⁻/⁻

B

% of mice with ocular disease

Age (months)

Mkp-1⁻/⁻/Il-10⁻/⁻
Mkp-1⁺/+/Il-10⁻/⁻
Mkp-1⁺/+/Il-10⁺/+/
Mkp-1⁻/⁻/Il-10⁺/+/

120
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<thead>
<tr>
<th>Feature scored</th>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation severity</td>
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</tr>
<tr>
<td></td>
<td>1</td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>Moderate</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Severe</td>
</tr>
<tr>
<td>Inflammation extent</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Mucosa</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mucosa and submucosa</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Transmural</td>
</tr>
<tr>
<td>Crypt damage</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Basal 1/3 damaged</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Basal 2/3 damaged</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Crypts lost; surface epithelium present</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Crypts and surface epithelium lost</td>
</tr>
<tr>
<td>Percent involvement</td>
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<td>0%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1%-25%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>26%-50%</td>
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<tr>
<td></td>
<td>3</td>
<td>51%-75%</td>
</tr>
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<td>4</td>
<td>76%-100%</td>
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**Table 4.1** Histologic Colitis Scoring Criteria
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<th>Feature scored</th>
<th>Score</th>
<th>Description</th>
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<td>Mucosal hyperplasia</td>
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<td>Normal</td>
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<tr>
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<td>1</td>
<td>Mild (epithelium up to twice normal thickness)</td>
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<tr>
<td></td>
<td>2</td>
<td>Moderate (epithelium between 2 and 4 times normal thickness)</td>
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<td></td>
<td>3</td>
<td>Severe (epithelium greater than 4 times normal thickness)</td>
</tr>
<tr>
<td>Goblet cell depletion</td>
<td>0</td>
<td>None (0% goblet cell loss)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Mild (1%-35% goblet cell loss)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Moderate (36%-70% goblet cell loss)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Severe (71%-100% goblet cell loss)</td>
</tr>
<tr>
<td>Lymphoid Aggregate</td>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td>Hyperplasia</td>
<td>1</td>
<td>Mild (small focal aggregates)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Moderate (small multifocal aggregates)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Severe (Large multifocal to diffuse aggregates)</td>
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</table>

**Table 4.2** Histologic IBD Scoring Criteria
**Figure 4.3** Deletion of *Mkp-1* on an *Il-10* null background exacerbates colitis. (A) Time course of the clinical score of colitis. *, *p*<0.05, compared to *Mkp-1*+/+/*Il-10*−/− group. ‡, *p*<0.05, compared to *Mkp-1*+/+/*Il-10*++ group. (n>10). (B) Histological intestinal inflammation scores. (C) Histological IBD scores. In B and C, the total inflammation or IBD scores represent the sum of the ascending, transverse, descending colons, and rectums. *, *p*<0.05, compared to *Mkp-1*+/+/*Il-10*−/− group. n=4.
Figure 4.3

A

![Graph showing average clinical score over age (weeks) with different genotypes indicated by symbols.]

B

![Bar graph showing average inflammation score with genotype comparison.]

C

![Bar graph showing average IBD score with genotype comparison.]

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Figure 4.4 Knockout of Mkp-1 exacerbates intestinal inflammation of Mkp-1\textsuperscript{+/+}/Il-10\textsuperscript{-/-} mice. (A) H&E staining of sections of colons from Mkp-1\textsuperscript{+/+}/Il-10\textsuperscript{-/-}, and Mkp-1\textsuperscript{-/-}/Il-10\textsuperscript{-/-} mice. (B) Distribution of T and B lymphocytes in the colon of double knockout mice detected by IHC with $\alpha$-CD3 and $\alpha$-B220 antibodies. (C) IHC analysis of rectums from Mkp-1\textsuperscript{+/+}/Il-10\textsuperscript{-/-}, and double knockout mice with $\alpha$-p-histone H3 antibody. (D) IHC analysis of occludin expression in colons of Mkp-1\textsuperscript{+/+}/Il-10\textsuperscript{-/-}, and double knockout mice.
Figure 4.4

A

Mkp-1^+/+//Il-10^−/−  Mkp-1^−/−//Il-10^−/−

B

Mkp-1^−/−//Il-10^−/−  Mkp-1^−/−//Il-10^−/−

α-CD3  α-B220

C

Mkp-1^+/+//Il-10^−/−  Mkp-1^−/−//Il-10^−/−

α-p-histone H3  α-p-histone H3

D

Mkp-1^+/+//Il-10^−/−  Mkp-1^−/−//Il-10^−/−

α-occludin
**Figure 4.5** Conjunctivitis in double knockout mice. (A) The conjunctiva in *Mkp-1^{+/+}/Il-10^{+/+}* mice was normal. (B) *Mkp-1^{-/-}/Il-10^{+/+}* and (C) *Mkp-1^{+/+}/Il-10^{-/-}* mice showed mild accumulation of mucus within the conjunctival space. (D) The conjunctival mucosa of double knockout mice was substantially thickened with occasional intraepithelial abscesses (as indicated by the arrow) and infiltration with inflammatory cells. Panels on the right are amplified views of the selected fields on the left. All sections were stained with H&E.
Figure 4.6 Double knockout mice develop blepharitis. Eyelids of (A) $Mkp-1^{+/+}/Il-10^{+/+}$, (B) $Mkp-1^{-/-}/Il-10^{+/+}$, and (C) $Mkp-1^{+/+}/Il-10^{-/-}$ mice were normal. (D) Eyelids of double knockout mice had markedly thickened epithelium and were infiltrated with inflammatory cells. Panels on the right are amplified views of the selected fields on the left. All sections were stained with H&E.
Figure 4.6

A
Mkp-1+/- II;10^7/+ 100X 400X

B
Mkp-1+/- II;10^7/+ 100X 400X

C
Mkp-1+/- II;10^7/- 100X 400X

D
Mkp-1+/- III;10^7/- 100X 400X

mast cell
lymphocyte
Figure 4.7 Mkp-1 deficiency exacerbates inflammatory responses. Splenocytes and combined lymph node mononuclear cells isolated from Mkp-1\(^{+/+}\)/Il-10\(^{+/+}\), Mkp-1\(^{+/−}\)/Il-10\(^{+/+}\), Mkp-1\(^{+/+}\)/Il-10\(^{−/−}\), and Mkp-1\(^{−/−}\)/Il-10\(^{−/−}\) mice were treated with LPS for 24 h. TNF-α and IL-6 production by splenocytes (A) and lymph node cells (B) were assayed by ELISA. Data are presented as means ± S.E. Presented are the representative results of at least three experiments. *, \(p<0.05\), compared to LPS-stimulated wild-type cells. ‡, \(p<0.05\), compared to Mkp-1\(^{+/+}\)/Il-10\(^{−/−}\) cells.
Figure 4.7

A

B

TNF-α (ng/ml)

TNF-α (pg/ml)

IL-6 (ng/ml)

IL-6 (pg/ml)

LPS

Mkp-1
+/
+/
-/
-/

II-10
+/
+/
-/
-/

-/+ 
-/+ 
-/+ 
-/+ 
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132
Figure 4.8 Knockout of Mkp-1 enhances LPS-induced Th-1 cytokine production in Il-10-deficient cells. Splenocytes and combined lymph node mononuclear cells isolated from Mkp-1\textsuperscript{+/+}/Il-10\textsuperscript{+/+}, Mkp-1\textsuperscript{+/−}/Il-10\textsuperscript{+/+}, Mkp-1\textsuperscript{+/+}/Il-10\textsuperscript{−/−}, and Mkp-1\textsuperscript{−/−}/Il-10\textsuperscript{−/−} mice were treated with LPS for 24 h. (A) IL-12 (p70) and IFN-γ production by splenocytes (left panel) and lymph node cells (right panel) were assayed by ELISA. (B) IL-12 mRNA levels in splenocytes (left panel), and combined lymph node cells (right panel) were assessed by Northern blotting, and qPCR, respectively. IL-12 mRNA signals were normalized to GAPDH and Hprt mRNA, respectively. Values in B (left panel) are expressed as fold increase relative to control. Values in B (right panel) represent expression levels relative to those in untreated wild-type cells. Data in A and B (right panel) are presented as means ± S.E. of at least three experiments. *, p<0.05, compared to LPS-stimulated wild-type cells. ‡, p<0.05, compared to Mkp-1\textsuperscript{+/+}/Il-10\textsuperscript{−/−} cells.
Figure 4.9 Mkp-1 deletion exacerbates Th1 cytokine production and enhances MAP kinase activities in Il-10 null CD4+ T cells. CD4+ T cells isolated from combined lymph nodes of Mkp-1+/+ /Il-10−/− and double knockout mice were stimulated by TCR cross-linking for the indicated times. (A) Cytokine production was assessed by ELISA. Values are expressed as means ± S.E from three independent experiments. *, p<0.05, compared to Mkp-1+/+ /Il-10−/− cells. (B) Levels of phospho-MAP kinases in the cell lysates were assessed by Western blot analyses. Presented are the representative results of at least three experiments.
Figure 4.9

A

Mkp-1+/+ /Il-10-/-

Mkp-1-/- /Il-10-/-

IFN-γ (ng/ml)

IL-2 (pg/ml)

IL-4 (pg/ml)

IL-17 (pg/ml)

Time (h)

B

Mkp-1+/+ /Il-10-/-

Mkp-1-/- /Il-10-/-

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<th>120</th>
<th>0</th>
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<th>120</th>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-ERK</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>p-JNK</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td></td>
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</table>
Figure 4.10 Cytokine levels and MAP kinase activities are elevated in double knockout mouse intestinal tissues. Intestinal tissues were harvested from double knockout and Mkp-1\(^{+/+}\)/Il-10\(^{-/-}\) mice and homogenized. (A) Cytokine contents were measured by ELISA. (B) MAP kinase activities were assessed using phospho-specific antibodies. Cytokine contents were normalized to total soluble proteins in the homogenates. Values in A are means ± S.E from 3 animals. *, p<0.05, compared to Mkp-1\(^{+/+}\)/Il-10\(^{-/-}\) mice. Data shown in B are representative results from 3 animals.
Figure 4.10

A

![Graph showing cytokine levels in different tissues for Mkp-1+/+Il-10−/− and Mkp-1−/−Il-10−/−](image)

B

![Western blot analysis of p-p38, p-ERK, p-JNK, and β-Actin in different tissues](image)
The immune system is a complex collection of specialized cells and biological processes within an organism that protect against disease by identifying and killing pathogens (non-self) and tumor (altered self) cells. The main function of the immune system is to mount a response against microbial insults and eliminate pathogens while sparing the normal tissues of the host. The immune system is broadly divided into two major components: the innate immunity and the adaptive immunity, which interact closely and synergistically in the host defense (2). The innate immune system is the first line of defense against pathogenic organisms and is essential for the mobilization and function of the various immune effector cells. The innate immune system also functions as a gatekeeper for mounting an efficient adaptive immune response (22). In this chapter we will focus on discussing the innate immune system and the signaling events mediated by the TLRs. Detection of microbial components by TLRs triggers conformational changes that lead to the recruitment of IRAK and TRAF-6 mediated by adaptor proteins MyD88 and TRIF. TRAF-6 can activate both the NF-κB and MAP kinase pathways. NF-κB is critical for the transcription of inflammatory response genes, including genes of various cytokines and chemokines. MAP kinases, including ERK, JNK, and p38, also regulate
the expression of many pro- and anti-inflammatory genes. Upon activation, MAP kinases translocate to nucleus where they phosphorylate and activate transcription factors such as AP-1, leading to altered gene transcription. MAP kinases, p38 in particular, also enhance cytokine production through post-transcriptional mechanisms. p38 phosphorylates and activates MK-2, which in turn phosphorylates TTP, leading to both enhanced cytokine mRNA stability and accelerated cytokine mRNA translation. p38 and JNK are also important in activating the adaptive immune response and are required for regulation of Th-1 cells and IFN-γ production. Mounting an aggressive immune response that is capable of eradicating invading pathogens is dependent on the presence of various positive regulators downstream of TLRs such as IRAK, TRAF-6, MAP kinases, NF-κB, and pro-inflammatory cytokines. However, following activation of these signaling pathways, it is imperative to subsequently deactivate them. This in turn will restrain the potentially devastating actions of the immunological system on the host, thus preventing self destruction.

In order to achieve this balance between activation and deactivation, the innate immune system is equipped with a variety of negative regulators which operate at multiple steps along the signal transduction pathways downstream of TLRs. Negative regulators modulate the duration and strength of the transduced signals and control the production of inflammatory cytokines (217). For example, TLR4 was shown to be transiently suppressed in response to repeated stimulation with LPS. This phenomenon is associated with endotoxin tolerance, which is a protective mechanism against endotoxic shock (218). In addition to the modulation at the receptor level, a number of anti-inflammatory proteins are also produced, such as IL-1 receptor-associated kinase
IRAK-M, suppressor of cytokine-signaling (SOCS)-1, IκB (inhibitor of NF-κB), IL-10, and MKP-1. IRAK-M is induced upon TLR stimulation and negatively regulates TLR signaling by preventing dissociation of IRAK and IRAK-4 from MyD88 and formation of IRAK-TRAF6 complexes (219). Thus, IRAK-M regulates TLR signaling and innate immune homeostasis (219). Cytokines activate the JAK-STAT pathway upon binding to their receptors. SOCS-1 is a negative regulatory molecule of the JAK-STAT signaling cascade, and is involved in negative regulation of LPS signaling pathways (220). IκB is a negative feedback regulator of NF-κB, and acts by binding to and sequestering NF-κB dimers in the cytoplasm, thereby preventing them from translocating to the nucleus where they activate gene transcription (141). Anti-inflammatory cytokines such as IL-10 are also produced to modulate the immune responses (217). The primary biological functions of IL-10 is limiting and terminating the inflammatory responses (196, 197) through blocking the secretion of a large number of pro-inflammatory cytokines.

In mammalian cells, MAP kinases are primarily inactivated by a group of dual-specificity protein phosphatases through dephosphorylation of the critical tyrosine and threonine residues of activated MAP kinases (73). In this capacity, this group of protein phosphatases, including MKP-1, may serve as pivotal feedback control regulators in the immune response during microbial infection. MKP-1 functions as a feedback control mechanism that governs the production of a group of cytokines by limiting the activation of MAP kinases (97, 98, 100).

Through the action of these inhibitory proteins, cells not only terminate the signaling cascade at the cell surface, but also switch off downstream mediators, thereby silencing the signaling pathways and ending the production of pro-inflammatory
cytokines. Similar to pro-inflammatory cytokines, the anti-inflammatory cytokine IL-10 is also controlled in a negative manner, either directly or indirectly, by MKP-1-mediated pathways. By inactivating both the pro- and anti-inflammatory responses, homeostasis is restored to the immune system, allowing its components to return to their normal state before subsequently encountering more pathogens.

**Triptolide** – Since excessive production of pro-inflammatory cytokines has been implicated in the pathogenesis of a number of inflammatory and autoimmune diseases, considerable efforts have been made over the years to modulate the immune system by interfering with the signaling pathways that control cytokine synthesis. *T. wilfordii Hook F.* is an herb that has been used for centuries in traditional Chinese medicine to treat rheumatoid arthritis. Triptolide is a major component of *T. wilfordii Hook F.* and has been shown to possess potent anti-inflammatory and immunosuppressive activities. It accounts for most of the efficacy of this herb (48). However, the exact mechanism(s) by which triptolide exerts its activities remain elusive. In addition to therapeutic properties, *T. wilfordii Hook F.* also exhibits a strong cellular toxicity (109, 110). Because of its severe toxicity, widespread medical application of this herb has been prohibited. The toxicity of triptolide itself and *T. wilfordii Hook F.* can be explained by our findings that triptolide exerts its activity early within the TLR signaling pathway and dramatically blocks the activity of NF-κB and other unrelated transcription factors. The fact that triptolide functions early in the inflammatory response pathway means that it lacks selectivity and it will affect various mediators within the pathway which translates into a
high incidence of side effects. Therefore, it is not surprising that triptolide exhibits strong cellular toxicity.

In spite of the toxicity of *T. wilfordii Hook F.*, it is still being used as an anti-inflammatory drug in traditional Chinese medicine. There are several explanations for this phenomenon. First, data from our microarray experiments clearly indicate that some genes, for example cytokines and chemokines, are more potently inhibited than others by triptolide. We have shown that triptolide selectively inhibits the expression of a subset of genes involved in the immune response. A commonality between many of these triptolide-inhibited genes is that their transcripts are relatively unstable. This suggests that transcripts of genes with the shortest half-lives (such as cytokines, chemokines, and immediate early genes) are more sensitive to the effect of this herb than house-keeping genes (such as GAPDH, and β-actin) that have long-lived transcripts. Water decoction or alcohol extracts of *T. wilfordii Hook F.* have been taken orally twice a day for 30 days (221). Upon ingestion of the first dose of the herb, the cytokine and chemokine genes (with unstable and short-lived transcripts) are highly sensitive to inhibition by *T. wilfordii Hook F.*, causing their mRNAs to decay. On the other hand, the house-keeping genes (with more stable and long-lived transcripts) are more resistant to inhibition by *T. wilfordii Hook F.*, which will only have minor effects on the stability of these genes. In the period between doses, the house-keeping genes are only decreased slightly, while the levels of cytokine transcripts are substantially decreased, because of their short half-lives. When another dose is ingested, the same pattern will be repeated. The net result throughout the treatment period is that house-keeping genes will be maintained near normal levels while genes involved in the immune response will exhibit
a cyclical pattern of peaks and troughs. Such speculation can explain the “selectivity” of *T. wilfordii Hook F.* and the therapeutic effect of this herb within a narrow therapeutic window.

Second, in traditional Chinese medicine, it is common practice for herbal remedies to be composed of a mixture of various herbs and other ingredients. Therefore, the anti-inflammatory herbal remedies are likely to contain many components in addition to triptolide. Some of these components might exert beneficial effects and neutralize or even counteract the toxicity of triptolide. This in turn may explain the benefit seen with these herbal remedies.

**MKP-1** – In contrast to triptolide, MKP-1 is an endogenous anti-inflammatory molecule. MKP-1 was the first MAP kinase-selective protein phosphatase discovered that dephosphorylates both the phosphothreonine and phosphotyrosine residues on activated MAP kinases (68). MKP-1 is an important negative feedback control mechanism that regulates the MAP kinases and plays a pivotal role in restoring homeostasis to the immune system. Upon detection of microbial components by TLRs, MAP kinases are activated. ERK, in turn, regulates MKP-1 expression by two mechanisms: by enhancing MKP-1 gene transcription and by phosphorylating MKP-1 and thereby increasing its half-life. By phosphorylating MKP-1 protein, MAP kinases can regulate the stability of MKP-1 protein. The MKP-1 protein in turn dephosphorylates JNK and p38, thus stopping the perpetuation of the inflammatory cascades and terminates cytokine production. Furthermore, MKP-1 functions in synergy with IL-10 to terminate the inflammatory response. IL-10 increases MKP-1 expression, further enhancing MKP-1-
mediated feedback control of the inflammatory cascade. MKP-1 in turn negatively regulates IL-10. In addition to their role in the innate immune system, MAP kinases have been implicated in T-cell development and function. For example, p38 activation is necessary for IFN-\(\gamma\) production by CD4\(^+\) and CD8\(^+\) T cells, and JNK activation is necessary for IL-4 production by T cells (222). Inactivation of p38 and JNK by MKP-1 inhibits T cell activation and the production of T\(_{h}\)-1 cytokines. MKP-1 thus acts as a negative regulator of both the innate and adaptive immune responses. MKP-1 negatively regulates both the pro- and anti-inflammatory arms of the inflammatory response thus restoring homeostasis to the immune system. This allows the immune system to return to its normal state and mount another response upon subsequent encounters with pathogenic organisms.

Reflecting the importance of MKP-1, several laboratories including ours have employed MKP-1 knockout mice and demonstrated a central role for MKP-1 in the restraint of innate immune response and the prevention of septic shock syndrome during pathogenic microbial infection (122, 166, 167, 174). Based on these findings, we speculate that deficiency of \(MKP-1\) in animals and humans would put these individuals in a disadvantaged position during evolution, because they would be more susceptible to septic shock at young age and develop inflammatory diseases. These susceptibilities likely would have prevented them from reaching reproductive age or put them in a less competitive status to pass their genetic materials to future generations.

We also demonstrated a dosage effect for MKP-1 in determining susceptibility to colitis. Loss of one copy of the \(Mkp-1\) gene increased the susceptibility of \(Il-10\) null mice to the development of colitis. This indicates that the suppression of IBD by MKP-1 is not
only dependent on the presence of a functional \textit{Mkp-1} gene, but also requires the expression of the \textit{Mkp-1} gene at a sufficient level. These findings further highlight the importance of appropriate MKP-1 regulation, whereby reduction of the amount of MKP-1 in a genetically susceptible individual by 50\% is sufficient to cause disease. Therefore, upregulating the levels of MKP-1 would have a protective effect against the development of such disease. These findings are clinically relevant to human disease and raise the possibility that MKP-1 may be a potential as therapeutic target in IBD. It would be of great interest to investigate whether patients with IBD harbor polymorphisms or mutations in their \textit{MKP-1} gene, have abnormalities in their MKP-1 protein, or have defective signaling pathways that control MKP-1 production.

MKP-1 is an attractive drug target; it is highly inducible and therefore has the potential for being manipulated. MKP-1 can fine-tune the immune system, a small increase (~50\%) in levels of MKP-1 can be beneficial in the management of inflammatory/autoimmune diseases. Efforts to modulate MKP-1 activity in a time-, magnitude-, and tissue-restricted manner could prove to be a fruitful venue for the treatment of many inflammatory diseases. Currently there are no pharmacological agents that selectively upregulate MKP-1.
REFERENCES


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