# INDUCTION OF INNATE IMMUNE RESPONSES BY PLANT-DERIVED PROCYANIDINS AND POLYSACCHARIDES

by

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April, 2012

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# ABSTRACT

Plants contain most of the basic metabolic systems utilized by mammals, but also contain unique structures to interact with self and with non-self biomolecules. It is of little surprise, that many of these plant biomolecules impact mammalian systems. Numerous plant products are used for treating human disease and are critical for the most fundamental aspects of medicine including pain control and cancer therapy. In addition to these drugs, plant products have been used for millennia to improve disease resistance. Our understanding of how these plants activate the innate immune system to fight off infection is tenebrous, with very little understood about receptor-mediated responses. The following studies elaborate upon our current understanding of two common, plant-derived compounds with innate stimulatory activity: procyanidins and polysaccharides. Procyanidins are a class of polyphenols and flavonoids. The research described herein shows that procyanidins directly activated γδ T cells to enter a primed state and stabilized select gene transcripts via ERK- and syk-mediated processes. The second class of plant products discussed are polysaccharides from Acai. The innate immune response induced by Acai polysaccharides was mediated by TLR4 and the phagocytic response, possibly mediated by Dectin-1. These studies have improved our understanding of host responses to plant products, which have implications for consumption of both foods and nutritional supplements.

# CHAPTER ONE

## INTRODUCTION TO DISSERTATION

Numerous plant products are described that activate responses in mammalian systems that are relevant to clinical medicine. The responses elicited by plant products span many aspects of biology including immune responses. This dissertation describes the innate immune responses induced by to two types of plant compounds, polysaccharides and oligomeric procyanidins (OPCs). Background information on our current understanding of innate immune responses relevant to these studies and how they are affected by plant products is provided in Chapter Two.

While both procyanidins and plant polysaccharides activate the innate immune system, it is important to recognize that there are distinct differences in both the cell types responding to these products as well as in the types of innate immune responses generated by them. Due to the differences in structure and the responses induced by these molecules, the chapters are split into sections on procyanidins and on polysaccharides. Chapters Three and Four focus on procyanidin responses, while Chapters Five and Six describe polysaccharide responses. This work primarily focuses on describing the *in vitro* activity and the mechanisms of action for these plant products. Work performed primarily by collaborators shows the relevance of these responses in disease models. The implications of these *in vivo* responses are discussed in Chapter Seven.

## Procyanidins

The work on procyanidins is a continuation of a project that started during my tenure as a technician and continued into work described in my Master's Thesis. This work began as a screening project using natural and synthetic compounds to identify γδ T cell agonists. As part of our results from this screen, we identified plant compounds, including procyanidins, as agonists for γδ T cells and other components of the innate immune system. Previous reports and background information on structures, plant sources, and activities of a variety of polyphenols, as well as their clinical applications, are published in the *Journal of Immunology*[1], *Critical Reviews in Immunology*,[2] and *Animal Health Research Reviews*.[3]

This research consisted of our initial observations of a priming state in human and bovine γδ T cells when treated with procyanidins from Cat's Claw and Apple Peel Polyphenol (APP). Important observations from this report include the ability of procyanidins to directly stimulate purified γδ T cell populations, an increased responsiveness of OPC-primed γδ T cells to TCR stimulation, and that the oligomeric procyanidins were the most active component of the APP preparation. In the *Critical Reviews in Immunology* article,[2] we provide background information on immunomodulatory polyphenols. We also show that defined dimeric procyanidins activate γδ T cells and that this is not due to toxic or antioxidant effects, since toxic concentrations of the monomeric subunits do not similarly activate γδ T cells. Finally, the *Animal Health Reviews* publication[3] compares the phenotype of procyanidin

activation with that of molecular products, such as the Toll-like Receptor (TLR)4 ligand, LPS. This priming phenotype is associated with expression of activation markers such as CD69 and Interleukin (IL)-2Rα, and cytokines, including GM-CSF and MIP-1α, but is not associated with robust proliferation or interferon (IFN)γ production in the absence of secondary stimuli. Combined, these papers provide a description of the cells activated by procyanidins and the structures that are able to induce the effects.

My efforts to decipher the mechanisms of this event are the focus of the procyanidin section of this dissertation. Chapter Three describes work done in collaboration with Katie Daughenbaugh that examines how part of this priming response may occur. In this Chapter, we discuss the ability of procyanidins to alter the transcript degradation rates of select cytokines, namely *GMCSF*. We hypothesized that these transcriptional regulatory mechanisms explain, at least in part, how the primed γδ T cell responds rapidly to secondary stimulation.

In Chapter Four, I provide an unpublished manuscript that addresses the potential sources of γδ T cell-activating procyanidins. While we previously showed that purified procyanidins, common in many plants, activate γδ T cells, a thorough analysis of common plant extracts or defined polyphenols was undescribed[2]. To characterize the polyphenols capable of mediating stability, I developed a GFP reporter cell line from the γδ T cell-like line, MOLT14. This reporter expresses a GFP transcript with the 3'UTR from *GMCSF*. Using this reporter, I was able to screen polyphenol species with established immunomodulatory activity. In this screen, I showed transcript stabilization

from cells treated with procyanidins, but not with other polyphenols. This indicates the responses to procyanidins are distinct from many common polyphenol agonists. This screen also describes the Acai seed as containing the most potent γδ T cell stimulatory activity, suggesting it may be useful as an alternative source for procyanidins.

In Chapter Four, I use this reporter assay to define the molecular mechanisms responsible for the procyanidin-mediated stabilization of transcripts. Previously, the stabilization phenomenon was found to be dependent upon ERK kinase (Chapter Three). Using this cell line, I confirm that the 3'UTR is in fact associated with procyanidin-mediated transcript stability. Furthermore, using this line as a screening tool, I show syk is an additional kinase involved in procyanidin-mediated transcript stability. Combined, these results indicate that the kinases, ERK and syk, are involved in the stimulation of innate immune responses by OPCs.

## Polysaccharides

The polysaccharides discussed within are those derived from Acai fruit. This work is an extension of the analysis of the polysaccharides from Yamoa™, which was performed in collaboration with Jodi Hedges[4] and correlates with many of the studies by Schepetkin *et al*.[5-8] Our first description of Acai polysaccharide (AcaiPS) activity was published in *PLoS ONE*[9] and is provided in Chapter Five. This work described the basic immune cell response to these polysaccharides. AcaiPS activated both myeloid and γδ T cells. When delivered to the lung, AcaiPS induced myeloid cell recruitment and IL-12 production. Furthermore, a unique advantage of Acai polysaccharides, compared to previously

reported polysaccharide preparations, is that they do not contain LPS, nor do they react in the limulus (LAL) test.

In Chapter Six, I provide an unsubmitted manuscript looking at the potential mechanisms of AcaiPS. In these experiments, we examine the TLR signaling pathways and the polysaccharide receptor Dectin-1 for their roles in these responses. Dectin-1 is able to bind the polysaccharide and may play a role in phagocytic signaling via syk and resulting in ROS production. The signaling response to AcaiPS is also dependent upon TLR4, suggesting that AcaiPS activates innate immune responses via multiple mechanisms.

Finally, in Chapter Seven, I provide a compilation of results measuring the therapeutic potential of these two plant compounds. These studies, performed primarily by collaborators, show the therapeutic effects of these plant products in infectious disease and in inflammatory disease. Procyanidins were shown to protect against intestinal damage during a DSS model of colitis and to reverse neutrophilia. Procyanidins were also shown to protect *in vitro* against dengue virus challenge and are being examined for potential *in vivo* therapeutics. The Acai polysaccharides also have *in vivo* applications. Acai polysaccharides induce a strong Th1 response and this was shown to be protective in *Francisella* challenge. These potential therapeutic modalities emphasize the importance of characterizing mechanisms leading to the unique immune responses provided by OPC and Acai polysaccharides.

# CHAPTER TWO

## BACKGROUND

The innate immune system is divided into three functions: Barrier function, Phagocytosis, and Inflammation. These functions are mediated by a network of innate immune cells. Interfaces between the host and the environment, such as the skin, mucosa, and intestine, highlight the interconnectedness of these three functions. First, a tight network of cells is present in these locations, which prevents pathogens from entering the system. These cells are also bathed in a variety of antimicrobial products including the following: dead cells and organic acids in the skin, saliva and mucus in the oral cavity, tears in the eyes, as well as flora and acids in the gut and skin, respectively. These all serve as the barrier arm of innate immunity. Next, underneath the top layer of cells forming this barrier are cells that actively sample the external environment probing for pathogens, and when found, endocytose/engulf and digest them, thereby performing the phagocytosis function. Finally, the third arm of innate immunology is the inflammatory response. Throughout these barriers are cells containing receptors to detect pathogens and induce immune programs to destroy them, either by altering the environment or recruiting other cell mediators to do so. These barriers must also regulate inflammatory responses since overt inflammation destroys the integrity of this barrier and exposes the host to the external environment. These three components of the innate immune response are tightly controlled and are continuously in process to protect the host from a pathogen before it gains a foothold in the host system.

To maintain these functions, cells of the innate immune system must sense damage and/or the presence of pathogen. This sensing of the external environment is mediated by a large number of receptors called Pattern Recognition Receptors (PRR), which allow the host immune system to rapidly control disease and tissue damage by recruiting appropriate cells to sites of inflammation.[10] PRRs recognize Pathogen-Associated- or Damage-Associated- Molecular Patterns, PAMPs or DAMPs, respectively.[10,11] The innate immune responses vary depending upon the type of external stimuli recognized. For example, tissue damage to the skin requires growth factors that mediate cell healing/wound closure and phagocytic cells to remove infectious agents. Alternatively, viral infection requires cytolytic cell recruitment to kill infected cells. The generation of appropriate responses based on PRR sensing enables the host to effectively control numerous types of insult.

Table 1.1 shows examples of immune cells with innate functions and references for more detailed information on the specific cell types. Of particular interest to early innate responses is the γδ T cell. Early in infection, many immune responses are regulated by the γδ T cell's ability to respond to host PAMPs and DAMPs, and thus eliciting appropriate responses.[12] This recognition occurs not only through the PRR, but also through the γδ TCR, which recognizes similar, unprocessed patterns .[13] The γδ T cell has functions in the tissues, where it directs recruitment of inflammatory cells, cytokine secretion, and interactions with the epithelial cells to properly maintain barrier function.[14] In addition, other subsets of γδ T cells found in circulation are rapidly recruited to sites of

inflammation to mediate responses. The ability of the γδ T cell to rapidly respond to conserved, unprocessed patterns and its location in the tissues make it a valuable contributor to the innate immune network, particularly at the mucosal surfaces.

Many plant products are taken as nutritional supplements for their inherent ability to activate innate immune responses and there are numerous examples of this type of activity.[2,15-18] However, few mechanisms are described for how plant products impact the innate immune system.[19,20] The literature defines some plant components that mediate PRR signaling and a few that point to direct engagement of cell receptors to induce this effect. These plant receptors include innate receptors such as Dectin-1 as well as other types of receptors such as 67kDa Laminin Receptor and Nrf2. .[19-21] These receptors and their ligands will be discussed in detail later. This dissertation will focus on two plant products in particular, the procyanidins and the polysaccharides, which are the focus of research preformed for this dissertation project. In this introductory chapter, I provide an overview of pattern recognition by the innate immune system and summarize the current literature describing the known innate immune responses to plant products.

Table 2.1 Innate Immune cells and their Location/Roles within the Host.

| Cell type | Location | Function |
|---|---|---|
| B1 B cells | Peritoneal cavity | Generate low affinity, broad spectrum antibodies[22] |
| Basophils and Eosinophils | Tissues | Histamine, Th2 responses, helminth clearance[23,24] |
| Mast cells | Surface tissues | Protease-containing granulocytes. Also regulate lung inflammation, healing[25] |
| Epithelial cells | Surface tissues | Barrier cell, homeostasis, Th2 licensing, limited responses to pathogen[26] |
| Myeloid DCs | Tissues, circulation (immature), LN (activated) | Prototypical DC population. Bactericidal responses Secretion of IL-12 |
| Plasmacytoid DCs | Circulating, peripheral LN | Antiviral responses, Secretion of Type I IFN |
| Neutrophils | Circulating, rapidly recruited to infection | Cytotoxicity, phagocytosis, NETs, PRR[27,28] |
| Monocytes | Circulating | Recruited to tissues to become macrophages/DCs |
| Macrophages | Tissues | Either M1: inflammatory phagocytic, microbicidal. or M2: anti-inflammatory wound repair, suppressive cytokines[29] |
| γδ T cells | Circulating, tissues | Inflammation, quiescence, wound healing. IFNγ, IL-17 |
| Invariant NKT cells | Tissues, skin, intestine | Recognition of lipids via CD1d, Th1, Th2, IL-17 profile varies with subset[30] |
| NK cells | Systemic, predominantly in circulation | Kill non-self cells based on improper expression of MHC[31], inflammatory: IFNγ[32] |
| RORγt$^{+}$ Innate Lymphoid Cells | Intestine, mucosal tissues | Pro-inflammatory, IL-17/IL-22 producing[32,33] |
| RORγt$^{-}$ Innate Lymphoid Cells | Tissues, Lung | Th2 cytokines[34,35] |

## Pattern Recognition Receptors are Key Mediators of Innate Immunity

In order for the innate immune system to function, it must first recognize changes to its environment. This is done, in large part, by the expression of Pattern Recognition Receptors (PRR) that sense common patterns associated with disease or cell damage. The importance of their role in immunity is underscored by the awarding of Bruce Beutler and Jules Hoffmann with the 2011 Nobel Prize in Medicine for their contributions to the characterization of the Toll-like receptor (TLR) class of PRR. The TLRs, as well as other classes of PRR, recognize a vast assortment of PAMPS and DAMPS. This provides a mechanism for the innate immune system to deliver a rapid response to a changing environment.

PAMP recognition is critical in many aspects of host immunity. These are usually the first signals to the host of infection and enable the rapid response required for preventing dissemination. Originally, the TLR was discovered in drosophila and its deletion from the fly resulted in spontaneous colonization with commensal microbes.[36] Translation to humans and plants identified Toll homologs[37,38] followed by a rapid expansion of the field leading to the characterization of many TLR receptors, ligands, and co-receptors as well as other PRR families with PAMP-recognition capabilities.

In addition to PAMPs, TLRs also recognize endogenous proteins, DAMPs, which are released during cellular stress. While the DAMP pathway remains poorly understood, it is activated by a combination of necrosis-induced events including the following: release of cellular components, complement cascade activation, and the degradation and release

of extracellular matrix (ECM) components.[39] The results of DAMP signals via TLRs provide initial defense at the wound site and mediate programs to clear damage.

Toll-like receptors are critical factors for host recognition of both PAMPs and DAMPs; however, recognition of patterns is not exclusively mediated by TLRs. Other PRR are involved in these responses, either as co-factors or as signaling receptors in their own right. In the following section, I provide an overview of the complex network of ligand binding, endocytosis, and receptor cooperation involved in TLR and other PRR-mediated responses.

The Toll-Like/IL-1 Family of Receptors

The principal class of PRR, and the best described, are the TLRs. These receptors share accessory proteins and cytoplasmic signaling motifs in common with the IL-1 family of cytokine receptors, and are thus classified in the same receptor family, Toll-like/IL-1 Family Receptors (TIR). The first identified member of this family was the cytokine receptor, IL-1R, which was used as a model system for the characterization of later TIR.[40] The current list of IL-1R-like cytokine receptors of this family include those for the cytokines IL-1, IL-18, and the recently identified IL-33 (formerly known as ST2). Additional IL-1-family cytokine receptors include IL-1Rrp2 (receptor for IL-1F6, IL-1F8, and IL-1F9) and orphan receptors with uncharacterized ligands (SIGIRR, TIGIRR, IL-1RAPL).[41] The IL-1Rrp2 and orphan receptors are not yet well characterized and are beyond the scope of this introduction, thus the reader is referred elsewhere for more

detail.[41,42] The cytokine receptors for IL-1, IL-18, and IL-33 induce very different immune responses and have assorted roles in immunity.

IL-1-Associated Receptors. The IL-1R complex recognizes both IL-1α and IL-1β cytokines despite the fact that these cytokines share little sequence homology (24%).[43] IL-1β is normally produced in response to inflammatory signals and the induction of transcription factors such as NFκB, whereas IL-1α is constitutively produced by many cells, although it too can be regulated. One unique function of IL-1α is that it can localize to the nucleus where it alters transcription. Also, due to its nuclear localization, IL-1α is released during necrosis/apoptosis and, thus, functions as a sensor for tissue damage.[44,45] These different expression patterns for the IL-1 cytokines provide them with unique roles in inflammation.

Although they are structurally dissimilar, IL-1α and IL-1β bind the same receptors and thus elicit nearly identical responses, which include inflammation and fever.[43] Engagement of these cytokines with the receptor, IL-1R, is regulated by a number of inhibitory molecules (Figure 2.1). First, the soluble non-signaling competitor, IL-1RA, binds to, but does not engage, IL-1R thus blocking cytokine docking. Secondly, IL-1 cytokine signaling can be inhibited indirectly by IL-1R2, which acts as a cytokine receptor, but does not contain the signaling domain, thus it sequesters cytokine. Finally, IL-1R inhibition is mediated by soluble receptors, either sIL-1R or sIL-1R2. Signal events at the TIR are mediated in the cytosol via the accessory protein IL-1RacP; which

engages MyD88, thereby leading to downstream signals that will be covered in detail later.

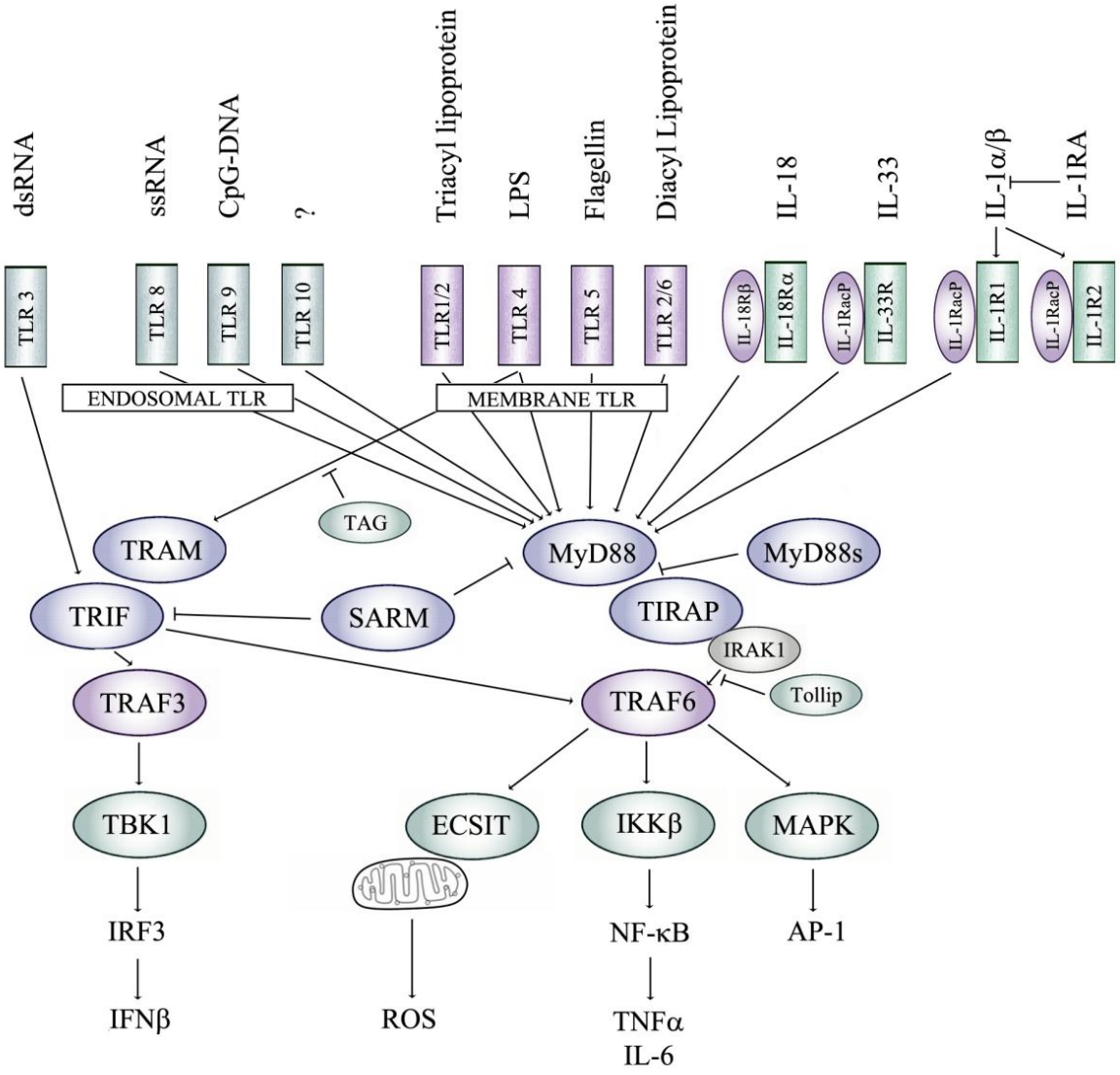
IL-18-Associated Receptors The IL-18R is similar to IL-1 in that it contains a cytokine receptor (IL-18Rα) and a signaling domain (IL-18Rβ). This signaling domain is similar to IL-1RacP for mediating engagement with MyD88. The IL-18R complex also contains an inhibitory sponge, similar to IL-1R2, named IL-18BP. Response to IL-18 cytokines is largely mediated by induced expression of IL-18Rβ, which is expressed under inflammatory cytokine conditions, including IL-12 and IL-23.[46] IL-18 typically promotes the generation of IFNγ, therefore it plays an important role in inflammatory responses to viruses and intracellular pathogens.

IL-33-Associated Receptors The IL-33 (a.k.a ST2) receptor was recently described. It shares the IL-1RacP adaptor that was previously discussed with IL-1R signaling.[47] IL-33R is expressed by lung cells, including mast cells and the recently-described nuocyte population of Innate Lymphoid Cells.[35] IL-33R signaling leads to the generation of Th2 cytokines.[48] Similar to IL-1α, IL-33 has a nuclear localization sequence and full-length, immature IL-33 is implicated in suppression of NFκB activity by interacting with NFκB binding of target DNA. This is one explanation for the reports that IL-33 is able to suppress TLR- and IL-1-induced NFκB activation.[49,50] In contrast, other groups report the induction of NFκB by recombinant IL-33.[48] Understanding the role of this cytokine in NFκB activation is important for deciphering how signaling through the TIR receptors often leads to different outcomes.

The other component of the TIR-family of receptors is the pattern-recognizing Toll-like receptors (TLRs). There are ten known TLRs in humans and twelve in mice. The human TLR receptors are numbered 1-10 and recognize many molecular patterns from gram-positive bacteria, gram-negative bacteria, viruses, and other parasites (Figure 2.1). In addition to recognizing PAMPS, TLRs are also able to recognize an expanding list of DAMPs. With the exception of some self-derived nucleic acids and anti-phospholipid antibodies, which are recognized by TLRs 3/7/8/9, most DAMPs are recognized by TLR2 or TLR4.[11] Examples of these endogenous ligands are shown in Table 2.1. This list is not complete, there are more putative DAMP/TLR associations that are not yet fully characterized.[11]

The downstream signaling of TLR/IL-1 ligand engagement is mediated through two possible adaptor molecules depending on the TIR. All TIRs signal through either the MyD88 pathway, the TRIF pathway, or, as in the case of TLR4, both. MyD88 is the most commonly used pathway, being utilized by all TIRs except TLR3. MyD88 activates the Interleukin-1 Receptor-Associated Kinase (IRAK) family of kinases, leading to TNF Receptor-Associated Factor (TRAF) 6 recruitment and activation. Other receptors are also involved, including Toll/Interleukin-1 Receptor Adapter Protein (TIRAP), which is required for bridging heterodimers and successful signaling of MyD88-associated events. The other adaptor molecule pathway, used by TLR3 and TLR4, is TIR-domain-containing adapter-inducing interferon-β (TRIF). TRIF utilizes an additional adaptor molecule, TRIF-Related Adaptor Molecule (TRAM) for TRAF6 activation, but additionally activates the transcription factor IRF3 through TRAF3

Figure 2.1 The Signaling Network of TIR-Containing Receptors




Toll-Like and IL-1-like Receptors mediate signaling through either TRIF or MyD88.[10,43,51,52]

Table 2.2 TLR Recognition of DAMPs

| Receptor/Ligand | DAMP structure |
|---|---|
| TLR-2 | |
| β-defensin-3 | Protein |
| HSP60, 70, Gp96 | Protein |
| HMGB1 | Protein |
| Serum Amyloid A | lipoprotein |
| Biglycan | proteoglycan |
| Hyaluronic acid | glycosaminoglycan |
| TLR-4 | |
| Fibronectin | Protein |
| HMGB1 | Protein |
| β-defensin-2 | Protein |
| HSP-60,-70,-72,-22,and Gp96 | Protein |
| Oxidized LDL | lipoprotein |
| Biglycan | proteoglycan |
| Heparin sulfate | glycosaminoglycan |
| Hyaluronic acid | glycosaminoglycan |
| Elastase | Protein |

and TBK1.[10] The fifth and final TIR-binding protein, Sterile-Alpha and Armadillo Motif-containing protein (SARM), is a negative regulator of TIR signaling.[10] Also able to inhibit TIR activity are splice variants of MyD88, sMyD88, which lack one or more signaling domains and act as competitive inhibitors for MyD88 signaling[53]. These TIR-binding adaptor molecules cooperate with the various TLR and IL-1R family members to initiate first stages of downstream of TIR ligation.

Under normal conditions, both TRIF- and MyD88-mediated signaling cascades lead to the activation of NFκB, MAPKs, and Reactive Oxygen Species (ROS) production.[52] Cytokines associated with TLR activation include TNFα, IL-6, and IL-1β, as well as chemokines, such as IL-8. In addition to these MyD88-associated responses, TRIF-

mediated responses also induce Type I IFN production via IRF3. It is important to note that these are the prototypical responses to TIR engagement and are not induced in all cells by all TIR ligands. In fact, although PAMPs, DAMPs, and IL-1 family members all signal through the same mediators, the end result of their engagement is often vastly different. For example, IL-1R and IL-18R engagement results in either IL-6/IL-1 or IFNγ-centric responses, respectively.[46] Furthermore, signaling through the same receptor, TLR4, causes different responses, as is shown by microarray analyses performed in neutrophils treated with the DAMP, HMGB1, or the PAMP, LPS[54]. This appears to be a common response with the TLR4 receptor, wherein TLR4 engagement by PAMPs generally leads to both a TRIF- and MyD88-mediated response, engagement with DAMPs engages only the MyD88 pathway.[11]

How this difference in gene expression from the same receptors occurs is still relatively unclear and is a source of contention. There is evidence through point mutation experiments in TLRs that DAMPs bind different regions of TLR than PAMPs,[55] yet others that indicate DAMPs and PAMPs utilize the same or nearby binding sites and therefore probably signal similarly.[11] Ultimately, the binding sites of the PAMPs and DAMPS may be a matter of semantics, since building evidence indicates that the largest factor in these differentiated signals is the use of different co-receptors. The engagement of many non-TLR PRR serve as co-receptors and affect recognition, endocytosis, and signaling of TLRs and thus appropriately tune the immune response.[11]

Non-TLR Pattern Recognition Receptors

The earliest described co-receptors for TLRs are the TLR4 co-receptors MD-2, LBP, and CD14. In fact, the first described TLR co-receptor, CD14, and the chaperone LPS-binding protein (LBP), were identified before TLR4.[56,57] CD14 itself does not have a cytoplasmic domain, and thus probably does not signal. Instead, CD14 acts as an LPS chaperone, mediating TLR4, as well as TLRs -1,-7, and -9, recognition.[58,59] This chaperone activity of LBP and CD14 requires a third molecule, MD-2, for presentation to LPS. In fact, TLR4 does not recognize LPS by itself, but rather the crystal structure for the MD-2/TLR4/LPS complex indicates that TLR4 contacts only MD-2.[60] These co-receptors, LBP, CD14, and MD-2 are all required for optimal response to LPS.[57,61,62]

Additional PRR are divided into functional families, prominent examples of which include the C-type Lectin Receptors (CLR), Scavenger Receptors (SR), and the cytosolic PRRs, Retinoic acid-inducible gene-I-like Receptors (RLRs) and Nucleotide Oligomerization Domain (NOD)-like Receptors (NLRs). Many of these PRRs possess their own signaling pathways, but also co-signal through TLRs. Shown in Table 2.2 are examples of these PRR and their known ligands. While many epitopes of these PRR overlap with the TLR family, others are situated to recognize unique patterns, either by cellular localization or by recognizing ligands not associated with other PRR. Importantly, responses to these PRR are not the same as TLR, providing combinatorial responses, such as endocytosis required for optimal innate responses to pathogen.

Scavenger Receptors. Scavenger receptors (SR) are lipoprotein receptors typically associated with binding of LDL and other lipid-containing moieties and mediating their removal from the host. Many of these receptors are also able to bind DAMPs or PAMPs and are involved in the resulting immune responses. The SR most often associated with TLR signaling is CD36. This Class B SR can recognize oxidized LDL,[63] serum amyloid A,[64] and thrompbospondin[65] on its own and induce signaling via Src-family kinases, MAPKs, and Vav exchange factors to mediate endocytosis, ROS production and IL-8 expression.[64,66] CD36 is also requisite for many TLR2 responses[67] and is required for the atypical formation of a TLR4/6 heterodimer.[68] In addition to CD36, other SR, including Macrophage Receptor with Collagenous domain (MARCO), the Class A, and other Class B SR, are described with PRR-mediated endocytosis.[69-71] Endocytosis is a common theme in SR-mediated events, yet the signaling responses due to SR are highly varied and are often coupled to other PRR receptor engagement.

C-type Lectin Receptors. C-type lectin receptors (CLR) contain at least one C-type lectin domain that enables them to bind carbohydrate structures. Although other lectin-binding receptors often have some PRR capabilities, the CLR are most commonly associated with PRR sensing and endocytosis.[72] This diverse set of receptors is divided into two groups: the membrane bound group (Dectin-1, Dectin-2, DC-SIGN, Mannose Macrophage Receptor, and MINCLE) and the soluble group, jointly called the collectins, (SP-A, SP-D, and mannose-binding lectin (MBL)). Together, these receptors recognize

many pathogen epitopes, particularly fungal-associated polysaccharide patterns, to induce innate immune responses.

Dectin-1 is the most studied of the CLRs and is critical for protection against many fungal pathogens. It is expressed in myeloid cells and on IL-17$^{+}$ γδ T cells.[73] Dectin-1 recognizes polysaccharide structures with β(1,3) glucan linkages and collaborates with TLR2/MyD88.[74] In addition to TLR2-mediated responses, Dectin-1 contains its own signaling domain and will activate syk/CARD9, as well as Raf-1, through independent pathways.[75,76] Syk activation leads to the activation of both the canonical (RelA) and non-canonical (RelB) NFκB pathways.[75] The second pathway activated by Dectin-1, Raf-1, leads to suppression of the RelB pathway, thus promoting c-Rel genes including IL-12-p35/-p40/IL-23-p19, IL-10, and IL-6.[75] These cause a unique inflammatory response to polysaccharides that are particularly prominent when coupled with additional TLR signaling events.[77]

Endocytosis plays a role in many of the Dectin-1 signaling mechanisms. Endocytosis of Dectin-1 is restricted to particulate antigen, and is required for optimal activation[78]. However, some components of Dectin-1 signaling are independent of endocytosis, including the activation of the caspase-8 inflammasome.[79] In neutrophils, Dectin-1-mediated endocytosis is accomplished, in part, via cooperation with CD11b/CD18.[72] Therefore, the phagocytic activity of Dectin-1, at least in some circumstances, requires cooperation with other receptors.

Dectin-2 is the signature receptor of its own family of CLR (Dectin-2, BDCA-2, DCIR, DCAR, Clecsf8, and MINCLE). Unlike the cytoplasmic domain in Dectin-1,

Dectin-2-like lectins (with the exception of DCAR, which is inhibitory) do not have a cytoplasmic tail capable of signaling. Instead, these motif-free receptors utilize the ITAM-containing FcRγ for the activation of syk and CARD9.[80] The Dectin-2-like lectin receptors engage signaling and endocytosis in response to different ligands. Dectin-2 recognizes high-mannose and α-mannan structures. MINCLE, in addition to binding pathogenic ligands, mycobacterial glycolipids, and α-mannose, binds to the endogenous protein SAP130, which is normally located in the nuclear compartment as a spliceosome component. Functional studies on Dectin-2 and MINCLE show that they are critical for fungal immunity through endocytosis/phagocytosis and the induction of Th1/IL-17 responses.

The Mannose Receptor (MR) or CD206 recognizes terminal fucose and mannose structures, yet does not have a definable signaling motif in its cytoplasmic tail. MR is primarily expressed in intracellular stores of macrophages and DCs, but can also be released after Dectin-1 signaling.[81] Most of MR's described activity is in the role of collaborating endocytic responses.[82] In spite of its ability to collaborate with other PRR for cytokine production *in vitro*, a clear role for MR in infections is lacking.[82,83] Therefore, beyond its role in endocytosis, which can be compensated for with numerous other phagocytic PRR, MR's function is still largely unknown.[83]

The soluble CLR, the collectins, are a group of soluble polysaccharide-binding proteins. The surfactant proteins A and D (SP-A, SP-D) reside in the lung and are associated with appropriately modulating immune responses to prevent overt inflammation and thereby maintain $O_2$ transfer to the bloodstream. The SPs block TLR

responses and sequester endotoxin to reduce inflammation. They also promote limited inflammation by inducing phagocytosis of numerous pathogens by phospholipid, carbohydrate, or lipid moieties.[84] These activities modify the immune response in the lung, presumably to produce an immune response suited to pathogen clearance and removal of non-pathogenic inhalants while still providing protection. Alternatively, MBL is a collectin found in circulation. It is produced by the liver, but is also found in intracellular stores. MBL mediates compliment via MBL-associated serine proteases (MASPs) and also initiates endocytosis, likely through CD91.[85] MPL also increases NFκB activity during TLR2/6 signaling events, through an as yet incompletely defined mechanism.[86]

Cytosolic PRR. The final groups of PRR are found in the cytoplasm. They include the Retinoic acid inducible gene (RIG)-I-like receptors (RLR) and the Nucleotide-binding Oligomerization Domain (NOD)-Like Receptors (NLR). These receptors form the functions of the TLRs, except that they are designed to recognize intracellular pathogens. The RLR recognize foreign nucleic acid by means of a DEAD box helicase/ATPase domain and thus are a PRR for genomic DNA or RNA viruses.[87] The NLR are likewise cytosolic sensors; however, they recognize bacterial peptidoglycans via CARD domains.[88] These cytosolic PRR play non-redundant roles in innate immunity since, unlike many PRR, they scan the cytosol for PAMPs.

Modulation of TLR Responses by Co-Factors

Co-receptors can modulate the TLR pathways in many ways.[89,90] First, they amplify or inhibit the formation of TLR-binding complexes in lipid rafts. Second, they modulate endocytosis. Third, they produce signaling pathways of their own that attenuate or amplify the responses of the TLRs. The most diverse TLR, TLR4, is one of the most studied TLRs for its ability to stimulate both MyD88 and TRIF pathways. It is also one of the most regulated and most influenced by co-receptors and provides for an excellent example of how these networks operate to fine-tune PRR-mediated responses.

In large part, the activity of TLR4 is regulated at the point of ligand binding at the cell surface by soluble and membrane bound chaperones and inhibitors. As previously mentioned, MD-2 is required for LPS binding, and its sequestration inhibits LPS, but does not inhibit DAMP-mediated signals.[91] The availability of soluble or membrane bound CD14 and LBP also impact the signal strength of the receptor. Conversely, TLR4 activity can also be inhibited. This is accomplished by soluble and membrane bound molecules. Soluble molecules such as surfactant proteins in the lung are examples of soluble factors that bind and block TLR4 ligand recognition. In addition, the membrane-bound receptor, MD1/RP105, is a competitive inhibitor for TLR4.[92] Combinations of these and other co-receptors modulate TLR4 recognition of ligand at the surface and thus modify the immune response.

TLR4 signaling can also be adjusted to favor the MyD88 or TRIF pathways. Modulation of the TRIF/MyD88 axis in TLR4 signaling is mediated by endocytosis of TLR4, which shifts signaling toward TRIF-centric pathways.[93] In addition to the

Table 2.3 Non-TLR Pattern Recognition Receptors

| PRR | Structures Recognized | Origin of the ligand(s) |
|---|---|---|
| Scavenger receptors | | |
| CD36 | Oxidized LDL, Thrombopoietin, PAMPs in the context of TLR2/6 | Endogenous lipidated products, TLR2/6 ligands |
| SR-A (CD204) | Oxidized/acetylated lipoproteins, LPS, environmental particles[69] | Endogenous, bacterial and environmental |
| Gp340 | Leucine-rich peptides[94] | Bacterial surface proteins |
| C-type Lectins | | |
| Dectin-1 | β(1,3)glucans[95] | Fungal, plant, and bacterial polysaccharides |
| MR (CD206) | Terminal mannose, fucose, or *N*-acetyl glucosamine. High-mannose PS and inflammatory proteins including myeloperoxidase[83] | Endogenous inflammatory proteins, fungal and bacterial polysaccharides. Viral glycoprotein. |
| DC-SIGN | ICAM-2/3, High-mannose and fucosylated structures[96] | Endogenous adhesion molecules, Fungal polysaccharide, viral glycoprotein |
| Dectin-2 | α-mannan, high-mannan | Fungal, dust mites, helminths, mycobacteria |
| MINCLE | U2 spliceosome component SAP130[97,98] | Endogenous and pathogen |
| Mannose-binding lectin (MBL) | Terminal mannose, fucose and N-acetyl-glucosamine. Mannan, TLR4[99],MD2, CD91[85] | Predominantly microbial sugars, but endogenous proteins and phospholipids as well |
| SP-A/SP-D | TLR2/4, SP-D: carbohydrates and phospholipids<br>SP-A: lipids[84,100] | Bacterial, viral, fungal products |
| NOD-like receptors | | |
| NOD1 | γ-D-glutamyl-mesodiaminopimelic acid and lipophilic peptidoglycan[101] | Gram negative and some gram positive bacteria |
| NOD2 | Muramyl dipeptide | Gram-positive and -negative bacteria |
| RIG-I-like receptors | | |
| RIG-I | Short dsRNA, 5'ppp-dsRNA, some U-rich 5'ppp-ssRNA[87] | Viral |
| MDA5 | Long dsRNA[87] | Viral |
| LGP2 | Predicted RNA species, may be inhibitory[87] | Unknown |

requirement for endocytosis, TRIF activation by TLR4 requires an additional accessory protein for activity, TRAM. There are two isoforms of TRAM, the full length version, which allows proper TRIF signaling through TLR4, and a splice variant, TAG, that acts as a negative regulator.[102] This network of endocytic and TRIF-centric adaptors enables TLR4 to differentiate between TRIF- and MyD88-associated signals depending upon co-receptor activity.

In addition to directly impeding TLR signaling cascades or skewing them toward other pathways, signaling events often up-regulate inhibitory proteins. The best example of this response is LPS tolerance, wherein repeated treatment with LPS leads to subsequent diminishing responses. This is caused by numerous mechanisms, which are still not fully elucidated, but it is mediated by inhibitors of TLR signaling including negative regulators SARM and Tollip (Figure 2.1)[103] as well as IRAK-M, SHIP-1, and A20.[104] Co-receptors also directly attenuate TLR signaling, such as SR-A-mediated TRAF6 inhibition.[105] How TLRs and co-receptors synergize remains an under-developed field. Nonetheless, the presentation and cross-activation of pathways appear to culminate in many unique inflammatory responses, presumably best suited to mediate regulation of disease.

PRR cross-talk can also have additive effects. One of the most robust instances of this occurs with TLR4 and Dectin-1. As mentioned previously, Dectin-1 induces numerous signaling pathways including the activation of syk/NFκB and Raf-1. Normally, during Dectin-1 or TLR4 signaling, NFκB activation is temporary and is rapidly shut down by feedback mechanisms. During Dectin-1/TLR4 co-stimulation

however, NFκB pathway is activated longer than with either alone.[77] Furthermore, the activation of Raf-1 by Dectin-1 promotes RelA-centric responses including the production of IL-10, IL-12 (-p35/-p40), and IL-23-p19 that is not appreciated with TLR4 or Dectin-1 alone. These events emphasize how PRR for a complex network of cooperation and inhibition to produce unique and potent responses.

Syk is a particularly interesting kinase in regards to TLR co-signaling. There is mounting evidence showing numerous roles for syk in mediating TLR responses. This kinase can have both positive and negative regulatory roles in TLR activation. Syk is required for the CD14-mediated endocytosis of TLR4.[61] Syk associates with the TLR4/MyD88 complex and is responsible for the phosphorylation of tyrosine on TLR4 during engagement. Inhibition of syk does not impact the production of most TLR4-mediated cytokines including TNFα, IL-6, IL-8, IL-1β, and MIP1α, but syk inhibition reduces the production of IL-10 and IL-12p40.[106] The roles of syk in attenuating TLR pathways remain unclear. One mechanism could be the increased production of IL-10 and autocrine suppression by this cytokine. However, syk also mediates the ubiquitination and proteolysis of MyD88 and TRIF via tyrosine phosphorylation.[107] These effects of syk on TLR signaling emphasize how PRR signaling is a cooperative process with large amounts of cross-talk between the adaptor molecules of these pathways.

The TLRs are expressed by many cells of the innate immune system, which allows for the sensing of a diverse repertoire of patterns in the surrounding environment. The complexity of this system is evident in the vast number of receptors, inhibitors, and co-

factors involved in the signaling process. All of these molecules cooperate to provide an appropriate response to a disease state. An often central part of this response is mediated by the γδ T cell. In addition to expressing a number of PRR including TLRs,[12] scavenger receptors,[108] CTLs,[73] and NLRs,[109,110] the γδ T cell also expresses a unique receptor, its TCR, that allows it to similarly sample the external environment and thus mediate rapid immune responses.

## The γδ T Cell Regulates Early Innate Immune Responses

γδ T cells were the first iteration of the T cell to develop during evolution and have numerous functions in mediating the host immune response, particularly in terms of early innate responses. Most γδ T cells promote inflammatory responses by producing either IFNγ- or IL-17-centric responses, which are important in the clearance of many pathogens. There are two general populations of γδ T cells, the circulating and the resident population. Tissue-resident γδ T cells make up the majority of the γδ T cells in the host and regulate barrier integrity through both pro-inflammatory and anti-inflammatory responses as well as wound healing. Alternatively, the circulating γδ T cells function as rapid responders that are recruited to sites of infection and mediate inflammatory responses for pathogen removal and mediate wound repair. This ability to produce a variety of signal responses is coupled with numerous germline-encoded innate PAMP and DAMP receptors including TLRs,[111-113] NLRs,[111,114] Lectin Receptors,[112,115] Scavenger Receptors,[115,116] (Table 2.4) and with the γδ TCR itself. These receptors

allow the resident, as well as the rapidly recruited γδ T cell, to sense and appropriately respond to early infections, tissue damage, or other malignancies. This section will discuss how the γδ T cell senses ligand, the changes this causes in the cell, and how these activation strategies enable this cell to play a critical role in the development and management of early immune responses.

Table 2.4 List of Innate Receptors Detected in γδ T Cells

| Myeloid cell/innate cell receptor | Reference |
|---|---|
| TLR1 | Hedges *et al*., 2005[111] |
| TLR2 | Hedges *et al*., 2005[111] |
| TLR3 | Hedges *et al*., 2005[111] |
| TLR4 | Hedges *et al*., 2005[111]<br>Lahmers *et al*., 2006[112] |
| TLR5 | Hedges *et al*., 2005[111] |
| TLR6 | Hedges *et al*., 2005[111] |
| TLR9 | Hedges *et al*., 2005[111] |
| Scavenger Receptors | Meissner *et al*., 2003[115]<br>Lahmers *et al*., 2006[112] |
| CD36 | Lubick and Jutila, 2006[116] |
| Mannose Receptor | Meissner *et al*., 2003[115]<br>Lahmers *et al*., 2006[112] |
| Galectins 1 and 3 | Meissner *et al*., 2003[115]<br>Lahmers *et al*., 2006[112] |
| Dectin-1 | Martin *et al*., 2009[73] |
| LOX-1 | M. A. Jutila, unpublished observations |
| CD11b | Hedges *et al*., 2003[113]<br>Meissner *et al*., 2003[115]<br>Lahmers *et al*., 2006[112]<br>Graff and Jutila, 2007[117] |
| CD14 | Hedges *et al*., 2003[113] |
| NOD2 | Hedges *et al*., 2005[111] |
| Numerous NK cell receptors | Lahmers *et al* ., 2006[112] |

Source: Jutila *et al*, 2008[3]

The γδ T Cell Priming Response

One of the primary functions of the γδ T cell is to function as a sentinel in the mucosal or epidermal surfaces. In this position, it must be able to rapidly respond to invading pathogen, but also balance anti-inflammatory roles so as not to attenuate barrier function. To address this balancing act, γδ T cells enter a pre-activated, or primed, state, allowing for a rapid response to additional stimuli when exposed to PAMPs.[3] This response is subtle compared to antigen recognition through its TCR.[1,2] Priming is characterized by changes in the cell that enable it to quickly react to additional antigen stimulation. The primed γδ T cell is induced by the recognition of PAMPs such as TLR agonists[111,116,118,119] or muramyl dipeptide[109] and, as recent studies in our lab indicate, by procyanidins.[1-3] The primed γδ T cell, though not overtly activated, is induced to generate cytokines including TNFα, TGFβ, GM-CSF, MIP1α, MIP1β, MCP-1, RANTES and IL-8[111,119] and to up-regulate inflammatory sensors and activation markers such as IL-2Rα and CD69.[111] In this state, the γδ T cell is able to recruit other cells and to robustly respond to secondary stimulation such as TCR engagement[119,120] or inflammatory cytokines.[1,120]

The Phenotype of γδ TCR Signaling

Although the γδ T cell expresses many germline-encoded receptors to sense the external environment, what separates γδ T cells from other cells of the immune system is the expression of the γδ TCR. The TCR is generated by the VDJ recombination system to produce a variable receptor for the recognition of antigen. Unlike the prototypical T

cells, which express the αβ TCR, the γδ TCR is highly clonal; specifically, there are only a limited number of VDJ recombinations actually observed in the peripheral γδ T cell population. Furthermore, the γδ TCR structure is more similar to an immunoglobulin molecule than to the αβ TCR, and this structure allows the γδ TCR to recognize unprocessed antigen.

Precisely what the γδ T cell recognizes with its TCR still remains largely unknown; however, identified ligands are largely DAMPs or PAMPs. An exception to this are the MHC-like ligands, T10/22, in mice.[121] Recognition of this MHC-like molecule by γδ TCR is atypical and independent of antigen presentation by T10/22. How this ligand is recognized and regulated γδ T cell activation remains unclear. Two hypotheses have been proposed for how T10/22-specific γδ TCRs are regulated. In the first, T10/22 protein expression can be induced and thereby signal γδ T cell activation. This may be the case for T10, which is inducible in many cells, yet the other ligand, T22, is constitutively expressed by many cell types.[122] The constitutive expression of T22 suggests another mechanism is involved in regulating T10/22 activation of the γδ TCR. Therefore, a second hypothesis is proposed that follows the observation that stress or infection alters the glycosylation of T10/22. It may then be that this alternatively glycosylated T10/22 is then sensed by the γδ TCR.[122,123] This second mechanism would follow the known functions of γδ T cell ligand recognition in that it would sense DAMP or PAMP-associated material.

Other known γδ TCR ligands include Heat Shock Protein 60 (HSP60),[124] mitochondrial ATPase,[125] and the non-vertebrate metabolites known as prenyl phosphates.[126] Also, Xi *et al*.[13] identified putative ligands for seven tumor-related epitopes, two hepatitis-associated epitopes, and two endogenous proteins released during cell stress (HSP60 and MSH2). These identified γδ T cell receptors underscore the hypothesis that γδ T cells recognize conserved self and non-self antigens in an innate-like manner.

One potential implication of TCR-mediated signaling is the development of IFNγ-centric cytokine production. This was originally shown during thymic development, wherein mice undergoing TCR signaling develop an IFNγ phenotype, but in the absence of ligand, they develop into IL-17-producing cells. This does not appear to be a permanent imprinting, since secondary stimuli (complete Freund's adjuvant) revert the γδ T cell to an IL-17-producing phenotype.[127,128] The generation of the IFNγ-producing phenotype is dependent upon CD27 co-stimulation, whereas IL-17 promotion is mediated by TLR signaling.[129] In addition to CD27, IFNγ-producing cells generally express CD122 but not CD25, suggesting a broad phenotypic change in IL-17 and IFNγ-skewed γδ T cells[130] similar to that seen with CD4 T cells. These studies indicate that the γδ T cell phenotypes ($IFN\gamma^+$, $CD122^+$, $CD25^-$, $CD27^+$ vs. $IL\text{-}17^+$, $CD122^-$, $CD25^+$, $CD27^-$) are managed by TCR-ligand or TLR-ligand interactions and implicate tissue distribution/ligand accessibility as driving factors in γδ T cell immune responses.

The ability to rapidly recognize conserved epitopes via TLR- or TCR-mediated events allows the γδ T cell to respond to pathogen or to tissue damage in an immediate and appropriate fashion. Early studies showed that function and location are segregated by γδ TCR variable chain expression, particularly in the well-studied mouse system. Since ligand interaction defines these phenotypes, how then does the γδ T cell, expressing the appropriate TCR to recognize ligand, migrate to its specified tissue? Recently, a mechanism to explain this phenomenon was described for the mouse Vγ5 or dendritic epidermal T cells (DETC). This mouse γδ T cell subset is produced early in development, traffics to the skin, interacts with unknown ligands,[131] and is skewed toward an IFNγ phenotype.[132] Selection for the DETC cell is mediated in early development by the thymocyte receptor, Skint1. In mice without Skint-1, the epidermis is populated by non-Vδ5 T cells and results in skin inflammation.[133] Skint-1 functions in propagating the appropriate DETC compartment by signaling the maturing DETC to suppress Sox13 and RORγt. This causes three different responses. First, Sox13 is a γδ TCR development factor, and its suppression stops TCR rearrangement at the first, Vγ5 stage. Second, RORγt suppression promotes Egr3 activity, leading to an IFNγ phenotype. Finally, Skint-1-deficient thymocytes are likewise deficient in transcripts for lymphotactin, which is required for DETC chemotaxis to the skin compartment.[132] These data show that the unique functions of the DETC population is a result of early development signals which impart the unique TCR and chemotactic signals required for appropriately populating the epidermis.[134]

Later in murine development, Sox13 is no longer suppressed, and the non-Vγ5 subsets are allowed to mature. Included in these are the circulating Vγ1 and Vγ4 subsets, which are critical for effective responses against many viral and bacterial infections. Other mouse γδ T cell subsets, include the tissue-associated Vγ6 (IL-17-skewed and associated with the reproductive tract)[135] and the Vγ7 subsets (IFNγ-skewed and associated with the intestinal tract)[136]. In both humans and cattle, similar, but less defined roles, are attributed to the subsets.

In humans, the circulating populations are Vδ1 and Vδ2, with the Vδ2 being more prevalent. Little is known about the resident populations, but include Vδ1 and Vδ3 cells.[137,138] Of the circulating populations, Vδ1 provides a more anti-inflammatory role and Vδ2 a more pro-inflammatory role.[139,140] Similar to mouse γδ T cells, these populations demonstrate functional plasticity and all will promote cytotoxic effects upon proper stimulation.[141-143]

γδ T cell subsets in bovine may be defined by Vγ chain expression;[144-146] however, these are not well defined functionally. Bovine γδ T cell subsets can also be defined by expression of a non-TCR surface glycoprotein, WC1. WC1 refers to the ruminant scavenger receptor, workshop cluster 1.[108] $WC1^{+}$ γδ T cells demonstrate a more inflammatory role in cattle, and the WC1- more anti-inflammatory.[113] The different gene expression patterns in bovine suggest that, similar to mouse and human, these γδ T cell populations have diverse roles in controlling different aspects of the innate immune response.

In summary, there are many aspects of γδ T cell biology that are described in certain species, but not in others. Some of these discrepancies may simply be that functional equivalents have not yet been confirmed in other species. The recent identification of SCART-1 and -2 in mouse γδ T cells suggests that exclusive expression of unique scavenger receptors on γδ T cells is not a unique phenotype to artiodactyla species (ruminants and pigs). This is an overlying theme for γδ T cell biology; each species seems to recognize and respond to unique DAMP and PAMP epitopes associated with host stress and/or infectious agents. Other receptors and innate immune cells also likely help to make up the differences in γδ TCR epitope recognition. This ability to innately recognize PAMP and DAMP allow these cells to quickly respond to their external environment, and thus form the basis of innate responses to many diseases.

γδ T Cells Protect the Host from Tissue Damage and Disease

γδ T cells are advantageous in many disease settings including early control and clearance of infectious agents, mediating cancer clearance, and maintaining epithelial integrity by preventing overt inflammation and promoting wound closure.[14,138] These responses are attributed to the functional plasticity of γδ T cells. They readily convert their phenotypes to produce appropriate cytokines or mediate healing, allowing them to respond appropriately.[12,131] This section provides an overview of these different aspects of γδ T cell activity during disease. While most studies are focused on the mouse, many of these responses translate to other species. These comparative studies show that the

ability of γδ T cells to protect against cancer and infection, as well as to regulate inflammation in the tissues, is common to all species.

Wound Healing and Suppression of Inflammation by γδ T Cells. γδ T cells are important in maintaining the health of epithelial barriers by controlling inflammation and promoting wound healing. Under normal conditions, mouse skin γδ T cells, DETC, migrate throughout the epidermis in a manner akin to surveillance and cluster in apparent recognition of self ligands via TCR.[131] Upon skin damage, either punch biopsy or burn damage,[134,147] DETC cells recognize damaged epithelial cells via the Coxsackie and Adenovirus Receptor (CAR) and Junctional Adhesion Molecule-Like protein (JAML) interactions to direct wound healing.[148] This leads to γδ T cell secretion of growth factors such as IGF and KGF, other remodeling signals, and the production of suppressive cytokines within hours of damage to rapidly close wounds and regain the integrity of this barrier.[134,149]

In addition to directly promoting wound closure by keratinocytes, DETC mediate deposition of the glycosaminoglycan, hyaluronan, into the epithelial tissues. This is directly synthesized by the DETC or induced in neighboring epithelial cells by KGF-1. As mentioned previously, hyaluronan release during tissue damage leads to innate immune signaling via TLR and CD44 pathways. In γδ T cell-deficient mice, hyaluronan deposition is diminished, leading to decreased macrophage recruitment during tissue damage.[150] Macrophages have numerous functions in sterile inflammation and in wound repair, including: cytokine production, ECM production, phagocytosis, and growth factor

secretion.[151] Therefore, the role of γδ T cells in wound closure also includes creating an environment to promote the recruitment of appropriate cell populations.

DETC not only promote wound healing, they also regulate inflammation via the production of cytokines to mediate a homeostatic environment. In γδ KO mice, but not wildtype or αβ/γδ double KO, a dermatitis-like phenotype is observed in some mouse strains.[152] This is associated with anti-microbial responses from the αβ T cell in the absence of DETC suppression and is probably regulated by multiple factors. Mechanisms for this DETC-mediated suppression include the synthesis of an anti-inflammatory splice variant of thymosin[153] and the IFNγ-promoting phenotype of the DETC.[132] Contrary to the long-held principle that IFNγ is inflammatory, and thus causes tissue damage, IFNγ is repeatedly shown to suppress inflammatory responses in the mouse and the human skin.[154-156] The requirement for DETC cytokine and growth factor production indicates the role for this cell in maintaining the health of the skin is multifaceted.

In addition to the skin, γδ T cells also play a role in maintaining intestinal homeostasis. Deregulation leads to the development of a group of diseases called Inflammatory Bowel Disease (IBD). The treatment of IBD is an important field for the medical community, with approximately 1.4 million Americans and 2.2 million Europeans suffering from the disease.[157] Unfortunately, the progression stages of Inflammatory Bowel Disease (IBD) are complicated and not well understood. Developed IBD can be associated with deregulated Th2 responses (ulcerative colitis) or deregulated

Th1/IL-17 responses (Crohn's disease), but the early stages of disease leading to these responses are only beginning to be solved.[158] The ability of γδ T cells to regulate inflammatory and suppressive cytokines, as well as to promote wound healing, indicates they may be central to many components of these diseases.

One mouse model for IBD, Dextran Sulfate Sodium (DSS), causes acute intestinal damage with symptoms including weight loss, rectal bleeding, diarrhea, and colon thickening and shortening. These effects are aggravated in γδ $^{-/-}$ and KGF$^{-/-}$ mice,[159] indicating that the γδ T cell plays a homeostatic role in preventing inflammation. Although it is unclear if the γδ T cell population generates KGF directly, γδ T cell-deficient mice show a reduced epithelial cell growth phenotype, suggesting that, if they do not produce the KGF directly, they at least regulate its production.[159,160] An alternate model for chemical-induced IBD shows similar γδ T cell regulatory roles and expanded upon earlier findings to show that γδ- T cell deficient mice spontaneously develop IBD and that adoptive transfer of γδ T cells into wildtype or γδ T cell-deficient mice ameliorates IBD.[161,162] Combined, these studies show that γδ T cells play a protective role in preventing intestinal damage.

In spite of the production of growth factors by γδ T cells in these models, it is unclear whether γδ T cells are beneficial to IBD. In fact, numerous IBD mouse models show that γδ T cells actually promote IBD phenotypes through the production of inflammatory cytokines.[163] Under normal conditions, the γδ T cell intraepithelial lymphocyte (IEL) population is resident and consists primarily of Vγ7 (60%) and Vγ1 (30%) T cells that

constitutively produce IFNγ in response to commensal bacteria.[136,164,165] During infection or inflammation, this can rapidly change to a γδ T cell population with a IL-17$^{+}$ phenotype associated with pathogen clearance[166] as well as IBD promotion.[167-169] It is currently unclear which γδ T cells promote, and which suppress, inflammation. There are examples of IL-17 and its associated cytokine, IL-22, playing a protective role in IBD,[170,171] and many showing the detrimental effects of IFNγ.[172] Since the γδ T cell can produce both of these cytokines, they may impact the different forms and stages of IBD. The ability of γδ T cells to promote cytokine production and secrete growth factors suggests that they have diverse roles in IBD pathogenesis.

Wound healing in other tissues is mediated by mouse γδ T cell populations although their mechanisms are not as well described as with skin or intestinal γδ T cell populations. In corneal lacerations of mice, the recruited γδ T cell population is important in mediating healing via IL-17 and IL-22 dependent mechanisms.[173] γδ T cells are also implicated in repair of bone damage,[174] and some lung γδ T cells regulate inflammatory responses from αβ T cells through IL-22 secretion.[175] These additional roles for γδ T cells in tissue repair and protection demonstrate a ubiquitous role for these cells in the many tissues that they populate.

The role of human γδ T cells in wound repair relies heavily upon implied results in the mouse. Unlike the mouse, where TCR subset expression is associated with tissue distribution, humans utilize a limited diversity of γδ T cell populations. Furthermore, γδ T cell distribution for many mouse subsets is mediated by waves leaving the prenatal

thymus, whereas no such population is described for humans. Although the human skin T cell population contains both γδ and αβ T cells, they produce growth factor responses similar to mouse.[138] Humans also show similar deregulation of γδ T cell populations during atopic dermatitis, showing there is likely some conserved function within these populations. Additional parallels can be drawn between the human and mouse models for IBD. Patients with IBD possess an increased number of γδ T cells compared to non-inflamed tissue suggesting a similar response.[176] Furthermore, human IBD is associated with large amounts of KGF and increased expression of KGF receptor in the intestinal tissues, indicating the mouse models correlate with human disease.[177]

The roles of γδ T cells in tissue repair are an interesting phenomenon. These cells are very capable of turning into inflammatory mediators, thus it may seem counterintuitive that they also have these protective properties. However, the nature of the resident population is to protect the epithelial barrier and therefore must mediate both inflammatory and anti-inflammatory functions as required. Some cytokines produced by γδ T cells, such as IFNγ and IL-22, appear to incorporate both inflammatory and homeostatic responses at barrier sites.[178] Future studies into how these cytokines determine signaling fates will likely provide a greater understanding of how the γδ T cell incorporates cytokine production with growth factor secretion to mediate host barrier integrity.

The Role of γδ T Cells in Cancer Prevention. Cancer is continuing to grow as a prominent cause of mortality in humans. In Americans, it remains the second leading

cause of death behind heart disease, but if trends continue, it will be the most frequent cause of death in a few years.[179,180] Therefore, it is one of the greatest studied diseases for immunotherapy. The progression of cancer, particularly solid tumors, relies heavily upon generating an immunosuppressive environment that prevents inflammatory cytokine production and infiltration of inflammatory cells. The gold-standard for tumor clearance is IFNγ, but the IL-17 inflammatory cytokine is also implicated in tumor destruction, with conflicting results. IL-17 does suppress classic $T_{reg}$ cell populations,[181] but also promotes angiogenesis.[182] Therefore, a key objective in cancer immunotherapy is to reverse the suppressive state of tumors and to reinstate the inflammatory mediator IFNγ and possibly IL-17.

γδ T cells are able to produce both of these inflammatory cytokines and are protective in many forms of cancer. In humans, both of the primary γδ T cell subsets, Vδ1 and Vδ2, have anti-cancer potential. The Vδ1 T cell recognizes stress-associated ligands, MICA/B and ULBP1-3, which are often up-regulated on cancerous cells. Alternatively, the Vδ2 T cells recognize phosphoantigens, mitochondrial ATPase, and express the MICA/B receptor NKG2D. These molecules, associated with infection and cell stress, are up-regulated on cancerous cells.[183] The Vδ2 T cell subset is particularly well studied for its ability to kill cancer cells *in vitro* and for its rapid expansion, both *in vitro* and *in vivo*, during bisphosphonate or prenyl phosphate treatment. However, clinical trials with Vδ2 T cell immunotherapy have thus far been disappointing.[184]

Both human γδ T cell subsets have restrictions that must first be overcome before they can be successfully utilized for therapy. Many Vδ2 T cells cannot penetrate solid

tumors, and those that can are $CD27^-$ and thus are associated with an IL-17-producing phenotype. Treatment with prenyl phosphates activates these cells, but then prevents their tumor infiltration.[185] Finally, repeated prenyl phosphate stimulation leads to attenuated responses.[186] The Vδ1 T cells are not readily expanded and become suppressive in the tumor environment, thereby promoting the growth of the cancer.[187] Due to these discrepancies in the activity of human γδ T cell populations, a large amount of work is being performed testing co-stimulatory factors to properly mediate tumor killing. Potential co-treatments for use with current γδ T cell agonists include TLR co-stimulation[12] and antibody co-treatment (rituximab, anti-CD20).[188] Unfortunately, until an appropriate ligand for Vδ1 T cells is identified, the Vδ2 population is probably the only viable γδ T cell candidate for these cancer therapies.

γδ T Cells Protect against a Broad Range of Infectious Agents. γδ T cells are protective in many instances of infection including bacterial, viral, fungal and parasitic infections. Provided in Table 2.2 is a synopsis of the infections associated with γδ T cell activity. This table only touches on the literature involving γδ T cell-associated immune responses to pathogens. IFNγ is the most common cytokine attributed to γδ T cells during infection; however, the pro-inflammatory cytokine IL-17 is also often produced by γδ T cells during infection, and in fact, the γδ T cell is the primary source of this cytokine early in many infections.[135] Importantly, these cells can play a role in many different aspects of the disease. In

addition to the cytokine responses attributed to γδ T cells, they are also directly cytotoxic and phagocytic.[189] Through combinations of these different

Table 2.5 γδ T Cell Responses during Infection

| Infectious agent | γδ T cell response |
|---|---|
| *Francisella tularensis Salmonella*, *Legionella*, *Coxiella burnetii*, *Rickettsia, and Listeria* | Expansion of γδ T cells up to 50% of blood pool[190,191] |
| *S. enterica* serovar Typhimurium | $CD8^+$ γδ T IELs clear bacteria via NKG2D[192] |
| *M. tuberculosis* | Required for optimal Th1 responses and granule formation in the lung[193]<br>Directly kill via perforin/granulysin[194] |
| *Borrelia burgdorferi* | Synergizes with DCs via TLR2 to increase early cytokine production: IFNγ, IL-17, IL-1β, IL-6, IL-12,TNFα, GM-CSF, RANTES (DC) and decrease bacterial burden |
| *Brucella abortis* | Kills *in vitro*, reduces dissemination and prevents intramacrophage replication of the bacteria[195] |
| West Nile virus | Increased viral titer/dissemination in $\gamma\delta^{-/-}$ mice[196].<br>Vγ1/Vγ4 axis mediates WNV clearance via DC maturation and IFNγ production [197,198] |
| Herpes Simplex virus-1 | Increased titer/lesions/mortality in γδ T cell-depleted mice[199]<br>Human γδ T cells increase inflammatory cytokines (IFNγ, TNFα, IL-8, RANTES and MIP1-α)during infection [200] |
| HIV | Pro-inflammatory cytokines and direct lysis of HIV-infected cells by human γδ T cells.[201] Human Vδ2 T cells are the first population depleted during HIV infection [202] |
| *C. albicans* | IL-17-producing lung γδ T cells protect against early infection[203] |
| *Plasmodium falciparum* | γδ T cells are the dominant source of IFNγ in early infection[204] |
| *Trypanosoma cruzi*, *Toxoplasma gondii*, *Cryptosporidium parvum* | Infection is controlled by γδ T cells[205-207] |
| *Nippostrongylus brasiliensis* (intestinal nematode) | γδ T cells increases function of goblet cells, thereby preventing infection and limiting egg concentration[208] |

effector mechanisms, the γδ T cell impacts a variety of distinct pathogen and other disease states, thereby emphasizing its plasticity and value to the early innate immune response.

## Recognition of Plant Products by the Innate Immune System

There are a number of different chemicals produced in the plant that can have effects on the human immune system, though they are not well documented. These are reviewed in detail elsewhere.[2] In this dissertation, I focus on three plant components with the potential to stimulate immune responses. First, I review the mounting evidence showing that antioxidant responses, while effective *in vitro*, have come under scrutiny of late. Then, I discuss two families of plant agonists that have relevance to this dissertation, the procyanidins and the polysaccharides. These sections describe the different receptor-mediated responses that plant products facilitate upon the innate immune response.

### Antioxidant Responses and the Role of Nrf2

One established assumption for the immune-stimulating effects of plants is that antioxidant compounds, common in plants, are involved in regulating immune responses. This assumption is based on their ability to remove oxidizing agents. Oxidants come in a variety of forms such as NADPH from macrophages and neutrophils as well as from numerous pollutants.[209] In the absence of oxidant scavenging molecules, oxidants cause debilitating damage to the cell, particularly DNA, and lead to apoptotic events and, at least *in vitro*, cancer.[210] These oxidants are controlled naturally in the host by self-

produced proteins including superoxide dismutase (SOD) and glutathione. These antioxidants are recycled by a network of enzymes, such as glutathione peroxidase to maintain a constant pool of oxidant scavengers available. Deleterious effects of oxidants, such as DNA damage, are observed when the antioxidant capacity of the cell is overwhelmed. To prevent damage, the antioxidant reserve of the cell is up-regulated in times of oxidant stress. Regulation of host antioxidant and damage control genes is managed by the Kelch-like ECH-associated protein 1 (Keap1)/Nuclear erythroid 2-related factor 2 (Nrf2) pathway, which induces the production of hundreds of genes containing an Antioxidant Response Element promoter sequence (ARE).

In addition to the hypothesis that many cancers are a result of oxidant-mediated DNA damage, many chronic inflammatory syndromes, such as IBD, are also associated with high amounts of oxidant damage. The systemic inflammation and diminished antioxidant activity[211] in IBD is considered to be a result of uncontrolled activation of mucosal T cells and ROS production.[212] ROS itself is detrimental to IBD by uncontrolled killing of cells and disruption of the mucosal barrier. ROS can generate inflammatory cytokine production and mediate apoptosis pathways, which further contribute to the pathology of the disease.[210,213]

Early studies suggested that these clinical manifestations of chronic disease and prevention of cancer could be controlled by limiting oxidant actions directly. These studies focused on using antioxidants, particularly those in plants as they are rich sources of these compounds. This oxidant scavenging ability is associated with phenolic rings that are common in many plant metabolites. Continuing clinical research on antioxidants

indicates that not all antioxidants or phenol-containing plant metabolites show a benefit in disease prevention,[214] and in fact suggest some non-plant antioxidants actually may increase mortality in low-risk patients.[215] Therefore, inhibiting oxidant activity directly does not appear to improve chronic diseases or prevent cancer.

As research on plant compounds continues, it becomes apparent that only some phytochemicals with antioxidant activity produce the previously described immune responses. These responses occur, not through preventing oxidative damage, but by directly triggering the host antioxidant pathway, Nrf2. The results of triggering this pathway are numerous. They not only increase antioxidant production and metabolism in the host, but promote many immune-associated activities.

Recent evidence suggests the "antioxidant response" is attributed to receptor-mediated signaling that is inducible by a limited number of plant compounds. One receptor-mediated event consists of Keap1/Nrf2/ARE pathway.[216] The Nrf2/Keap1/ARE transcriptional system is comprised of the sensor, Keap1, and the transcription factor, Nrf2, which promotes transcription of Antioxidant Response Element (ARE)-containing genes. During normal conditions Keap1 sequesters Nrf2, either by sequestering it to the outer membrane[217] or by mediating its degradation via ubiquitination.[218] During oxidative stress, the formation of oxidative products disrupts this interaction, releases Nrf2 to translocate to the nucleus, and leads to Nrf2-mediated ARE transcription.

Activation of Keap1 occurs via many products including oxidative products, $H_2O_2$, and redox cycling intermediates, as well as toxins, including N-iodoacetyl-N-biotinylhexylenediamine and tert-butylhydroquinone.[216] In addition to oxidative products,

some plant compounds, including curcumin, silymarin, quercetin, and Epigallocatechin Gallate (EGCG) are able to activate the Nrf2 system and lead to ARE activation.[219-222] Genes classically regulated by the ARE include enzymes designed to protect against oxidative damage and increase the safe removal of radical ions. These responses include: oxidative damage (NQO1, glutathione synthesis genes, peroxireductases, thioredoxin, and others); glycation (aldoketo reductase/dehydrogenase); metabolic stress (glucose-6P dehydrogenase, transketolase, transoxidase); lipogenic stress (sterol binding protein-1, fatty acid synthase); and clearance of damaged protein (proteasome proteins, autophagy). These responses lead to increased oxidant scavenging and to stress-associated responses designed to protect the cell.

In addition to up-regulating components protective against oxidative stress, Nrf2 also has effects on the immune response, which is typically anti-inflammatory. $Nrf2^{-/-}$ mice present with a spontaneous multiorgan autoimmune inflammation, are more susceptible to EAE, show increased mortality during LPS challenge, and are more sensitive to an OVA-challenge model for asthma. Nrf2 suppresses inflammatory responses via multifaceted gene transcription. Part of its effects come from the increased transcription of the anti-apoptotic protein, bcl-2, and presumably reduced tissue damage.[223] Another mechanism is the promotion of Th2 cytokine expression by an, as of yet, unidentified pathway[224] which thereby dampens overt inflammation. The combined observations of inflammation in $Nrf2^{-/-}$ mice and the roles of Nrf2 in suppressing inflammation show immunosuppressive functions of Nrf2 in addition to its role in directly controlling oxidative damage.

The ability of select plant metabolites to activate the Nrf2 oxidant response system may explain some of the positive benefits of these supplements in chronic inflammation. Recently, a meta-analysis of polyphenol supplement studies for cancer showed that of all the compounds tested, only quercetin, an Nrf2 agonist, demonstrated significant benefit.[214] The success of the Nrf2-activating molecules led to the characterization of synthetic Nrf2 agonists and early reports with these compounds show promise in alleviating chronic inflammation.[225]

There is currently a dichotomy in studies using antioxidants to treat chronic inflammation. The Nrf2 agonists show therapeutic potential in chronic inflammation, while general antioxidant products often have a neutral or even negative impact. Other "antioxidants" are described that do not activate Nrf2, and, in fact, induce very different immune responses.[1,226-228] Among these antioxidants are the oligomeric procyanidins and the 67LR agonist, EGCG, which are covered later in the chapter and may have opposing roles to the Nrf2 agonists; indeed they may intensify inflammatory conditions.[1,226-228] Therefore, use of values such as the Oxygen Radical Absorbance Capacity (ORAC), which describes, simply, the ability to scavenge free radicals is clearly inappropriate and perhaps detrimental. However, before replacing these values, much more information must be gathered on the immune effects of phytochemicals and the receptors involved in mediating their immune effects. Our research indicates other receptors are involved in the recognition of phytochemicals, and that they are part of the innate immune pattern recognition system (PRR). The next section will detail what is known of this evolving

system of host receptors for sensing many different molecules of their external environment.

## Innate Immune Responses to Polyphenols with a Focus on Procyanidins

Observations of plant product-induced responses to the human host number in the hundreds of thousands with journals solely dedicated to characterizing these compounds including: *Journal of Medicinal Plants Research, Fitoterapia, The Journal of Ethnopharmacology, Planta Medica,* and *Phytomedicine*. Polyphenols are some of the most often described compounds in the scientific literature. These compounds form a massively diverse group of plant metabolites with a minimum structure of multiple phenol rings and at least two hydroxyl groups. These are formed by either the shikimate or acetate pathways, with other metabolites often enzymatically attached. This expansive grouping of plant metabolites is further broken down into sub classifications including flavonoids, phenolic acids, coumarins, furocoumarins, lignans, stilbenoids, xanthones, styrylpyrones, quinones, phloroglucinols, and many others. The diversity of these molecules is enormous and as may be expected, has numerous effects on mammalian hosts.[2]

A common effect described for plant polyphenols is their ability to impact the immune system; however, these responses are highly variable. In addition to the aforementioned antioxidant/Nrf2 pathway that is stimulated by a variety of polyphenols, other immunostimulatory polyphenols are defined. There are at least two distinct responses to plant polyphenols in addition to the Nrf2 agonists, but due to the vast

number of different structures that polyphenols can form and the wide array of either inflammatory or anti-inflammatory responses that polyphenols can generate, there are likely even more. To identify unique responses, we must measure the ability of polyphenols to activate discrete immune pathways, and ideally identify the receptors mediating these responses. Thus far, two distinct pathways induced by polyphenols are described. These include the anti-inflammatory polyphenol EGCG and the oligomeric procyanidins (OPCs).

Of particular interest in the arena of anti-inflammatory research is the green tea polyphenol, EGCG, and its recently identified receptor, 67kDa Laminin Receptor (67LR). Originally described in 2004,[229] multiple groups have now confirmed that binding of EGCG to 67LR mediates signaling events including lipid raft clustering,[230] induction of eEF1A (tRNA chaperone),[19] and suppression of both TRIF and MyD88-mediated TLR signaling.[231,232] At least part of the TLR inhibitor effects are due to up-regulation of the activation of the TIR inhibitor, Tollip.[232] Tollip inhibits IL1R, TLR2, and TLR4 by inhibiting the TIR domain and also directly binds IRAK1, inhibiting phosphorylation (See Figure 2.1).[51] This explains the inhibitory effects of EGCG on the MyD88 pathway, but EGCG is also reported to inhibit the TRIF pathway.[231] It is currently unclear if TRIF is likewise attenuated by Tollip or if other TIR inhibitory pathways are engaged by 67LR signaling.[51] Further studies into this receptor-mediated signaling mechanism are thus required. The identification of this pathway now provides a potential mechanism for observations going back decades that green tea polyphenols demonstrate anti-inflammatory effects. Continued research in this area will improve our

understanding of this pathway, and the identification of the receptor improves the prospect of developing synthetic molecules that do not suffer from the limitations of EGCG, most notably poor solubility.

In addition to EGCG, many reports exist of plant polyphenols capable of modulating TLR-mediated signals. Provided in Table 2.4 and Figure 2.1 are additional examples of studies describing plant polyphenols that impact TLR signaling. These results could have implications in both regulating inflammation and in improving vaccine delivery, depending upon whether TLR activity is promoted or suppressed.

Table 2.6 Polyphenols with TLR-Modulating Activity

| Compound | Class of molecule | Cellular response |
|---|---|---|
| Triptolide | diterpene | Decreases TLR2/4 expression and NFκB nuclear localization |
| EGCG | catechin | Increases Tollip expression via 67LR signaling[232]<br>Inhibits TRIF via TBK1 suppression[231] |
| Curcumin | Curcuminoid, carbonyl-linked polyphenols | Inhibits oligomerization of TLR4 and NOD2 |
| Sparstolonin B | Isocoumarin/xantone | Inhibits MyD88 association with TLR2 and TLR4[233] |
| Oligomeric procyanidin | Flavonoid, proanthocyanidin | Pre-treatment inhibits LPS-induced NFκB activity.[234,235] Separate culture or LPS co-treatment promotes NFκB activity[236 237] |

One of the more commonly studied polyphenol groups are the oligomeric procyanidins. These compounds are found in many plant sources including cocoa, apple peels, grape seeds, and more[2]. Traditional medicines and herbal supplements are rich in OPCs and these molecules are shown to stimulate immune responses. Functional

responses to OPCs in experimental models support their use as a therapeutic agent. First, the γδ T cell population in the gut mucosa expands in mice[238] and rats[239] fed OPCs. This indicates a phenotypic change in the gut mucosa when stimulated with these γδ T cell agonists. Second, a reduced *Salmonella Typhimurium*-induced disease severity is observed in mice treated with APP (Hedges, unpublished observations). Finally, mice fed APP during a DSS-induced model of colitis demonstrate less severity.[240] These ability of OPCs to modulated these infectious and inflammatory diseases has clear implications in medicine; however, our understanding of how these phytochemicals, found in traditional medicines, as well as common foodstuffs, impact the host is largely unknown.

A large amount of data have been collected on the safety[241-243] and bioavailability[244-246] of OPCs from these plant sources. In fact, rats tolerate APP at concentrations more than 10 times higher than the recommended human intake.[2] Upon consumption, OPCs ranging in size from monomers to tetramers enter the bloodstream. The presence of these procyanidins is observed within minutes of injestion, peaks at 120m, and are absorbed approximately 24h later.[246] These results demonstrate the safety and the bioavailability of OPCs, suggesting they may serve as safe therapeutics for immune stimulation.

Attempts to characterize the signaling mechanisms of OPCs have had limited success. These polyphenols activate innate immune cells in a manner apparently independent of Nrf2[247] and do not appear to impair TLR signaling by the mechanisms of EGCG, curcumin, triptolide, or Sparstolin B. In fact, the reports on OPCs show very conflicting responses (See Table 1.6). By some reports, OPCs induce a mildly pro-inflammatory

phenotype.[1-3,226,227,236,237,248-250] This pro-inflammatory signaling response is characterized by increased cytokines for IL-1β and GM-CSF. Furthermore, unlike the inhibitory properties of other polyphenols, OPCs, particularly the larger oligomers, increase the cytokine response to the TLR4 agonist, LPS during co-culture; when treated with OPCs and LPS, human PBMCs produce more IL-1β, GM-CSF, TNFα, IL-10, and IL-6 compared to LPS alone.[236] In direct contradiction to these studies, other reports indicate that OPCs can diminish the cytokine response to LPS stimulation (Table 1.6).

Table 2.7 Examples of Pro-Inflammatory and Anti-Inflammatory Responses to OPCs

| Cell Source | Treatment | Result |
|---|---|---|
| Pro-inflammatory studies | | |
| Human PBMCs | 3 day culture with OPC fractions | Increase in TNFα production in trimeric and larger OPC fractions[237] |
| Human PBMCs | 8h culture of PBMCs (mRNA)<br>3 day culture of PBMCs (protein) | Increase in IL-1 expression during OPCs culture.[226] |
| Human PBMCs | OPC treatment alone or in co-culture with LPS | Increase in IL-1 and GM-CSF without LPS co-stimulation. LPS amplified cytokine production[236] |
| Anti-inflammatory studies | | |
| Human cell lines | 1h OPCs (B1, B2), followed by LPS and IFNγ treatment | Decreased expression of CXCL10, IL-8, TNFα, IL-1β, COX2[234] |
| Caco-2 | 30m OPC (hexamer), followed by TNFα | Reduced NFκB signaling (nuclear localization, DNA binding) and iNOS[235] |
| THP1 monocytes | 30m OPC (B2), followed by LPS | Suppression of LPS-induced COX2 [251] |
| THP1 monocytes | 4h OPC pretreatment | Decrease in LPS-induced ROS production[252] |

The discrepancies in these studies may be a result of kinetics and the amount of coumpound used in the assays. Many of the studies detailing the suppression of LPS-induced cytokine responses first treat the cells with OPCs for 30m to 1h before providing the stimulus.[234,235,251-253] TLR signaling can be inhibited in a variety of ways after pro-inflammatory signaling, including blockade of the TLR receptors by molecules, such as sMyD88 or Tollip, as well as further downstream at the level of NFκB, which can be suppressed in a number of different ways[254,255] and varies by the type of cell signaling.[256] These examples may partially explain why the anti-inflammatory phenotype is observed only under certain temporal culture conditions; however, this does not explain all observations. Terra *et al.*[257] show that while pretreatment with OPCs is the most effective form of suppression, long-term (19h) simultaneous co-culture of OPCs with LPS/IFNγ decreases prostaglandin E2 ($PGE_2$) production to 40-50% of LPS/IFNγ alone.[257] These reports indicate that the majority of the suppressive effects of OPCs, particularly on the NFκB pathway, are attributed to pretreatment modification of the cell.

Most of the anti-inflammatory responses seen from procyanidin appear to impact the NFκB pathway. It is unlikely that this is a result of classic self-regulation of NFκB by the transcription of IκBα [258] since OPCs do not activate NFκB.[1] Mackenzie *et al.*[228] demonstrated that dimeric procyanidin, as well as monomeric forms, inhibit NFκB via direct binding in a cell-free system. They further show that pretreatment of Jurkat cells with these procyanidins decreased IKKβ phosphorylation and NFκB/DNA binding. Importantly, this cannot fully explain the effects of OPCs since monomers, which do not

induce an inflammatory response,[1,237] bound to NFκB.[226,237] Thus an alternate system, not involving direct inhibition of NFκB or NFκB self-regulation, it likely the cause of this phenomenon. Regardless, of how this NFκB suppression occurs, the responses to OPCs are distinct from the previously described 67LR- and Nrf2-pathways, indicating they utilize a novel mechanism for their activity.

In earlier reports, I contributed to the characterization of OPCs[1,2,238,239] with immunostimulatory properties similar to the mildly pro-inflammatory responses described by others. In these studies, we show that OPCs from apple polyphenol extract (APP), grape seed, or Cat's Claw induce a priming-like response in γδ T cells.[1,3] During OPC culture, cells of bovine or human origin become activated to increase the expression of CD69 and IL-2Rα as well as become more responsive to cytokine-induced proliferation.[1] Similar to PAMPs, OPCs also increase the expression of chemokine transcripts, *Mip1a* and *Gmcsf*.[117] In addition to these subtle priming-like changes induced by OPCs, we found that APP-primed human Vδ2 T cells were substantially more responsive to TCR stimulation.[1] These results demonstrate OPCs induce a mild inflammatory response, similar to PAMP-induced priming. This has implications for generating a γδ T cell priming response in tissues that are refractory to TLR signaling.

Innate Immune Responses to Plant Polysaccharides

Polysaccharides are technically carbohydrate polymers consisting of two or more monosaccharide units. Many small polysaccharides, containing two sugars such as fructose and lactose, have roles in short-term energy storage. Other, relatively small

polysaccharides (two to ten units) are commonly bound to proteins, creating *O*- or *N*-linked glycoproteins. These smaller polysaccharides are generally referred to as oligopolysaccharides to discriminate them from massively larger polysaccharides containing numerous repeating units. These large polysaccharides, henceforth simply called polysaccharides in accordance with general terminology, have many roles in biology. For example, amylose and glycogen are used for storage of energy. In athropods, exoskeleton is composed of the polysaccharide, chitin. In plants, the polysaccharide cellulose provides scaffolding and structure. Finally, glycosaminoglycans make up a large portion of the extracellular matrix. Polysaccharide diversity is attributed to the types of monosaccharide subunits incorporated and branching. Each saccharide subunit can branch to form (1,4), (1,6), or (1,3) linkages to other saccharides. This potential to generate multiple linkages or to incorporate numerous monosaccharides enables the formation of complex biological structures.

Mammalian polysaccharide diversity is rather limited. With the exception of oligosaccharides (i.e. lactose and glycosylation products) and chitin in arthropods, polysaccharides are limited to glycogen for energy storage and the glycosaminoglycans (GAGs). The five glycosaminoglycans are heparin sulfate, keratin sulfate, dermatin sulfate, hyaluronic acid, and chondroitin sulfate. They are generally linked to proteins to form proteoglycans (with the exception being hyaluronic acid) and are distributed throughout the body in extracellular matrices.[259] These GAGs are recognized as DAMPs by some of the TLRs, presumably as part of the complement and ECM degradation cascades induced by necrosis.[39] In addition to TLRs, hyaluronic acid also engages CD44

on lymphocytes leading to wound repair and inflammation.[260] On top of these endogenous responses to polysaccharides, the mammalian immune system has adapted to recognize non-self polysaccharides as PAMPs.

Other kingdoms contain polysaccharides with unique structures that can be recognized by the immune system. The most studied polysaccharides in the context of infectious disease are the fungal polysaccharides and the C-type lectin receptors. As shown in table 2.3, many fungal polysaccharide epitopes are ligands for C-type lectins including β(1,3)glucan linkages, α-mannan, etc. The recognition of these is crucial for mediating proper immune responses to many fungal pathogens including numerous *Candida* species, *Aspergillus fumigatus*, and many more.[82] These receptors are, in fact, more important for mediating immune responses to fungi than are TLRs in many cases.[82]

In addition to polysaccharides from pathogens, the innate immune system recognizes many plant-derived polysaccharide structures, leading to the induction and modification of innate immune responses. A review from Schepetkin and Quinn provides examples of polysaccharides from 35 plant species that are able to activate macrophages.[261] These authors conclude that polysaccharides activate many inflammatory pathways of the macrophage including ROS production, NFκB activation, and subsequent cytokine release as well as MAPK activation. Thus far, receptors implicated in mediating these immune responses include the polysaccharide receptors including C-type lectins (Dectin-1, and MMR), complement receptor (CD11b/CD18), TLR receptors (TLR2, TLR4, TLR6), and the co-receptor CD14. Schepetkin and Quinn also note that these responses could be induced by contaminating PAMPS and that strict control of contamination, in

particular endotoxin, is necessary. Therefore, this section will discuss only those studies performed with proper controls, such as polymyxin-B treatment of the PS, endotoxin testing by LAL, or studies in TLR4 knockout/unresponsive mice.

The C-type lectins Dectin-1 and MMR recognize polysaccharides containing β(1,3)glucan and mannose-containing linkages, respectively. Both of these receptors mediate endocytosis, although MMR does not contain a signaling domain, and thus it may serve primarily in a endocytic role.[83] Alternatively, Dectin-1 does have signaling properties (described previously) in addition to its role in endocytosis.[75,79] Polysaccharides from *Ganoderma lucidum*[262] and barley[21] are able to bind the Dectin-1 receptor and signal via the C-type lectin-associated syk pathway. Tada *et al.*[21] performed detailed studies using purified, soluble β-glucans from barley in a Dectin-1/syk/CARD9/Bcl10 transfection of 293T cells to demonstrate the requirement for Dectin-1 in these responses. Therefore, C-type lectins are involved in at least some innate cell responses to plant polysaccharides.

The other phagocytizing receptor associated with plant polysaccharide responses is CD11b/CD18 heterodimer (a.k.a. Mac-1 or Complement receptor 3). This lectin-binding receptor is associated with complement-mediated endocytosis, but it can also bind a diverse repertoire of polysaccharide structures including β-glucan, mannose residues, and modified monosaccharides.[263] In order to bind these structures, the Mac-1 receptor must first enter an active conformation. This activated state is mediated by G-coupled receptors[264] or by Dectin-1,[72] but is not directly activated by β-glucans[72] as originally thought.[265] The activation of Mac-1 by plant polysaccharides therefore requires a two-

step process; first, the cell must experience a signaling event to activate Mac-1, and then Mac-1-mediated endocytosis occurs. The reports of Mac-1 binding to plant polysaccharides are not unexpected[266] since, once activated, Mac-1 will bind numerous sugars. In these same studies on *Angelica gigas*, it was also identified that responses were partially attenuated by CD14 blockade.[266] Since CD14 is most often associated with TLR signaling, in particular TLR4, it is not surprising that TLR4 signaling was later found to be involved in *Angelica gigas* polysaccharide immune responses.[267] This suggests a mechanism for polysaccharide recognition involving TLR4-mediated activation of the Mac-1 receptor and subsequent endocytosis by the Mac-1 complex.

Other polysaccharide preparations are described with TLR agonist activity. The most commonly described in plants is TLR4[268] [269,270] [271] [269,270] although TLR6 is required for response to *Tinospora cordifolia* polysaccharide.[272] The method by which TLRs are engaged by plant polysaccharides is unclear. Clearly, it is not by typical LPS structures since polymyxin B treatment does not remove activity in the majority of these responses. The TLR4 receptor is very promiscuous in its ligand recognition. Ligands shown to bind TLR4 include endotoxin, fungal polysaccharides,[273,274] heat shock proteins, fibrinogen, proteoglycans, fimbriae,[275] and nickel.[276] As mentioned earlier, TLR4 recognition of LPS is mediated by at least three co-receptors: MD-2, LBP, and CD14. The plant polysaccharide fraction of *Platycodon grandiflorum* is likewise dependent upon the co-factors LBP and CD14; however, MD-2 was not tested.[277] This report did not exclude potential, low-level endotoxin in these studies contributing to the polysaccharide response. Therefore, a more controlled analysis of these findings is in order before

attributing co-receptor usage to these or other TLR4-mediated plant polysaccharide responses.

Similar, non-lipidated polysaccharides from mushroom and bacterial polysaccharides are also ligands for TLR4 activation.[278,279] Therefore, we can look at these polysaccharides for structural similarities that may identify a conserved structure that enable them to be recognized by TLR4. One TLR4 ligand is the capsular polysaccharide from *Klebsiella*. This TLR4 agonist polysaccharide has a characterized structure consisting of a trisaccharide repeats of glucose-glucoronic acid-fucose with extensive pyruvation of the glucoronic acid and acetylation of the fucose residue. Importantly, the ability of this polysaccharide to engage TLR4 is dependent upon one or both of the pyruvate/acetate modifications.[279] Since plant polysaccharides can also be rich in fucose and often possess these modifications, a similar motif may be involved in the recognition of some of these structures by TLR4.[261]

It is clear from the different responses induced by plant polysaccharides that they trigger innate cell activation via different mechanisms. In addition to the different cytokine and inflammatory output by these cells, different signaling mechanisms appear to be utilized. Mac-1 appears to be required for some responses,[266] but not for others.[272] TLR engagement appears to likewise differ. Therefore, individual polysaccharides have the potential to mediate their effects through a variety of TLR and/or polysaccharide receptors.

The activation of these PAMP-like responses by plant polysaccharides is commonly studied in macrophages, since they express most of the TLRs and PAMP receptors. In

addition to macrophages, many other immune cells contain PRR, and reports of plant polysaccharides activating B cell and dendritic cells are also reported.[267,271] In fact, B cells may recognize *Acanthopanax koreanum* and *Astragalus membranaceus* polysaccharides by BCR, as well as TLR,[280,281] suggesting a B-cell restricted mechanism for some polysaccharides. In reviewing the current literature on plant polysaccharide-induced responses, it becomes apparent that there are many potential responses induced by these complex molecules. The diversity of plant polysaccharide receptors, as well as their responding leukocyte populations, may explain these different immunologic outcomes. Therefore each of these polysaccharide preparations may be uniquely suited for developing targeted modifications to the immune response.

## Limitations to our Understanding of Plant-Induced Innate Immune Responses

The identification of receptor/ligand mediated interactions in plant product-mediated immune responses is critical for developing them as therapeutics. The characterization of some products as Nrf2/ARE ligands, as well as the identification of 67LR as the receptor for EGCG, will expand of our understanding of these responses two-fold. First, the identification of these ligands will allow the characterization of ligand/receptor binding models. This will enable the testing of similar structures for activity and provide a target for the development of synthetic receptor agonists. This is particularly promising for the EGCG receptor, LL67, since EGCG is poorly soluble in water. The second benefit to identifying the ligands involved in mediating these responses is that this simplifies the

characterization of signaling networks stimulated upon receptor engagement. Since many of these plant compounds are tested *in vivo*, researchers have a head start on potential therapeutic avenues to be pursued.

The generation of suitable immune responses requires an appropriate early activation of the innate system to first identify, then control, and ultimately produce the correct adaptive immune response for protection. Inappropriate activation of the innate immune system exacerbates disease. This can be the result of unwarranted inflammation in the case of autoimmunity or the suppression of suitable inflammatory responses during infection. For example, the induction of a Th2 immune response during fungal or viral infection promotes dissemination.[282] *Vice versa*, a response appropriate for viral clearance increases tissue damage and promotes helminth colonization.[283] Other polarizing conditions such as IL-17/IFNγ,[198] or Th17/$T_{reg}$[284] bias, will likewise promote or deter proper disease resolution. Many pathogens have evolved mechanisms to modulate the switch to opposing immune responses themselves.[285,286] The fact that these pathogens attempt to divert appropriate immune responses emphasizes the importance of a proper immune response for pathogen clearance. For these reasons, a thorough understanding and characterization of the innate immune programs stimulated by plant products is critical before they can be appropriately prescribed for therapeutic use. A clear understanding of plant-induced immune responses not only provides a potential therapy for a specific disease, but will also prevent misuse and potential exacerbation of disease.

Truly defining the activity of any immune-stimulating product requires an understanding of every aspect of its activity including: *in vivo* and *in vitro* cell activation, receptor recognition, and internal signaling. Our understanding of how polysaccharides and OPCs activate the immune system does not meet all of these requirements. Similar to the recent characterizations of receptor/ligand interactions of the Nrf2- and LL67-agonists, the identification of the receptors for these plant agonists would greatly advance our understanding of how innate immunity is affected by these molecules.

As part of my dissertation, I worked to pinpoint the mechanisms of the OPC- and polysaccharide-mediated responses with the eventual goal of characterizing the receptors mediating their activity. The following chapters discuss the results of this research. Both of these plant products are associated with TLR/IL-1 mediated signaling, in particular the MyD88 pathway. However, as is common with TIR signaling, their induced responses vary greatly. These results emphasize the fact that plant products can elicit very different changes to the innate immune system. The use of nutritional supplements should therefore be taken with great care, so as to provide the correct immune response and not an oppositional one. To make this possible, we must first better understand the complexities of innate immune stimulation by plant products.

# CHAPTER THREE

## CONTRIBUTION OF TRANSCRIPT STABILITY TO A CONSERVED PROCYANIDIN-INDUCED CYTOKINE RESPONSE IN γδ T CELLS

### Contribution of Authors and Co-Authors

Manuscript in Chapter Three

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Contributions: Designed experiments.

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## Abstract

γδ T cells function in innate and adaptive immunity and are primed for secondary responses by procyanidin components of unripe apple peel (APP). Here we investigate the effects of APP and purified procyanidins on γδ T cell gene expression. A microarray analysis was performed on bovine γδ T cells treated with APP; increases in transcripts encoding *Gmcsf*, *Il8*, and *Il17*, but not markers of TCR stimulation such as *Ifng*, were observed. Key responses were confirmed in human, mouse, and bovine cells by RT-PCR and/or ELISA, indicating a conserved response to procyanidins. *In vivo* relevance of the cytokine response was shown in mice following intraperitoneal injection of APP, which induced production of CXCL1/KC and resulted in neutrophil influx to the blood and peritoneum. In the human γδ T cell-line, MOLT-14, *GMCSF* and *IL8* transcripts were increased and stabilized in cells treated with crude APP or purified procyanidins. The ERK1/2 MAPK pathway was activated in APP-treated cells, and necessary for transcript stabilization. Our data describe a unique γδ T cell inflammatory response during procyanidin treatment and suggest that transcript stability mechanisms could account, at least in part, for the priming phenotype.


## Introduction

In this study we took advantage of three different model systems (mice, cattle, and humans) to define conserved responses of γδ T cells towards plant-derived procyanidins. Microarray analysis of sorted bovine γδ T cells showed that a number of myeloid cell

cytokines, including *Il8* and *Gmcsf*, were up-regulated after APP treatment, confirming a PAMP-like γδ T cell priming response during procyanidin culture. Furthermore, mice injected intraperitoneally with APP displayed neutrophil influx and elevated CXCL1/KC (mouse equivalent of IL-8) levels in both the peritoneum and the blood, affirming the relevance of the expression of these cytokines *in vivo*. To test the impact of procyanidins on the stability of these AURE-containing transcripts, we used the human γδ T cell line, MOLT-14. Both *IL8* and *GMCSF* transcripts were stabilized in cells treated with either APP or purified procyanidin (PC1), and this event likely occurred via the ERK1/2 MAPK pathway. Together, our data suggest that a transcript stabilization mechanism is activated in APP-treated cells and could account, at least in part, for the γδ T cell priming response since transcript stabilization could enable the cell to rapidly produce large amounts of cytokines in preparation for secondary stimulation or insult.

## Materials and Methods

### Cell Culture

MOLT-14 cells (DSMZ, Brunswick, Germany) were maintained at a density of approximately $1.5x10^6$ cells/mL in complete RPMI (Mediatech, Manassas, VA, USA) containing 10% fetal bovine serum[113] (Hyclone, Waltham, MA, USA) at 37°C and 10% $CO_2$. Twenty-four hours prior to any experiment, cells were collected by brief centrifugation at 500x*g*, then suspended at $1.5x10^6$ cells/mL in serum-free medium, X-VIVO-15 (X-VIVO; BioWhittaker, Walkersville, MD, USA), in six- or twelve- well tissue culture plates (BD Falcon; Becton Dickinson, Franklin Lakes, NJ), depending on

the experiment. Primary bovine PBLs were obtained as previously described[1]. Briefly, freshly collected PBMCs were prepared by Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA) density gradient and monocytes were removed using anti-CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Monocyte depletion was confirmed by FACS (FACSCalibur, BD Biosciences). For transcript stabilization studies, cells in X-VIVO were treated with APP (Apple Poly®, Littleton, CO, USA) at 54μg/mL, recombinant human TNFα (PeproTech, Rocky Hill, NJ, USA) at 50ng/mL, recombinant bovine TNFα (Thermo Scientific, Waltham, MA), PC1 (Phytolab, Vestenbergsgreuth, Germany) at 50μg/mL, or PBS for 4h, then with actinomycin D (Sigma-Aldrich) at 5μg/mL. At the time-points indicated in the figures, cells were harvested from the wells and transferred to centrifuge tubes, then collected by centrifugation for 5m at 500x*g*. Supernatant fluids were removed and discarded, and then RNA was extracted from the cell pellets using the Qiagen RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. For studies using the U0126 inhibitor, cells in X-VIVO were treated with PBS or APP at 54μg/mL for 2h, then U0126 (Cell Signaling, Danvers, MA, USA) at 20μM was added and cells were incubated for an additional 2h, followed by treatment with actinomycin D as described above. For splenocyte culture, spleens were removed from WT (C57BL/6) or γδ T cell-deficient animals and were disrupted with ten strokes of a Dounce homogenizer in 10mL X-VIVO medium. Suspensions were poured through a 70μm filter to remove clumped cells and were plated in twelve-well plates at a density of $2x10^6$ cells/mL in X-VIVO.

Mouse Splenocyte Priming Assays

Carboxyfluorescein succinimidyl ester (CFSE)-labeled BALB/c splenocytes were prepared as above and cultured in X-VIVO medium with PBS or PC1 (15μg/mL) for 24h. Culture supernatant fluid was then replaced with fresh medium containing 1ng/mL rhIL-2 and cultured for an additional 72h. Cells were then stained for γδ TCR (GL3) and analyzed by FACS.

Microarrays

Microarray experiments were performed as previously described.[112,287] Bovine PBMCs were collected from two different calves and γδ T cells were stained with GD3.8 (pan γδ T cell mAb) and sorted using a FACS Vantage (Becton Dickson, Franklin Lakes, NJ, USA) to purities greater than 97%. Sorted cells were rested overnight in X-VIVO, and then stimulated with either PBS or APP (70μg/mL) for 4h. RNA was extracted and analyzed on an Agilent Bioanalyzer (Agilent Technologies, Wilmington, DE, USA) to assess integrity. RNA was then amplified and used to probe Genechip® Bovine Genome Arrays (Affymetrix, Santa Clara, CA, USA). Identical experiments were performed using cells from two different calves (four total arrays). The intensity of each gene on the APP array was normalized to its corresponding spot on the PBS array for each specific calf. Only genes determined to be present in all four arrays using the Gene Chip Operating Software program (Affymetrix) were used for further analysis. The fold-change in gene expression over PBS from each calf was then averaged, and those with a 3-fold or greater increase after treatment with APP were included in the analysis in Table

1. Microarray data was submitted to GEO and is accessible using the series number GSE13321.

Real-Time RT-PCR

RNA was used in reverse transcription reactions using random hexamer primers and SuperScript III enzyme (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The resulting cDNA was used in quantitative PCR experiments using SYBR green reagent (Applied Biosystems, Foster City, CA, USA) and primers specific for 18S (Forward: 5'-TCGAGGCCCTGTAATTGGAAT-3' and Reverse: 5'-CCCAAGATCCAACTACGAGCTT-3'), *GMCSF* (Forward: 5'-GCAGCCTCACCAAGCTCAAG-3' and Reverse: 5'-TCTTTGCACAGGAAGTTT-3'), *IL8* (Forward: 5'-TTCAGAGACAGCAGAGCACACA-3' and Reverse: 5'-GGCCAGCTTGGAAGTCATGT-3'), beta-actin (Forward: 5'-AAGGATTCCTATGTGGGCGA-3' and Reverse: 5'-TCCATGTCGTCCCAGTTGGT-3'), and *JUN* (Forward: 5'-CAGAGTCCCGGAGCGAACT-3' and Reverse: 5'-CCTTCTTCTCTTGCGTGGCT-3'). For mouse experiments the primers used were specific for beta-actin (Forward: 5'-CCTAAGGCCAACCGTGAAAA-3' and Reverse: 5'-GAGGCATACAGGGACAGCACA-3'), *Gmcsf* (Forward: 5'-GGTTCAGGGCTTCTTTGATGG-3' and Reverse: 5'-CCTGGGCATTGTGGTCTACAG-3'), and for *Cxcl1/Kc* (Forward: 5'-TGCTAGTAGAAGGGTGTTGTGCGA-3' and Reverse: 5'-TCCCACACATGTCCTCACCCTAAT-3'). Reactions were performed on a 7500 Fast

Real-time PCR System (Applied Biosystems) in triplicate, and data were normalized to *18S*/*18s* or beta-actin to obtain relative transcript levels. Each individual experiment was performed a minimum of three times, and data are presented as average ± standard deviation.

ELISA Analyses

CXCL1/KC protein was detected in the blood and IP fluid of mice immunized with APP using the Quantikine KC Immunoassay kit (R & D Systems, Minneapolis, MN, USA). IL-8 was detected in supernatant fluids from MOLT-14 cells treated with APP using the Quantikine IL-8 Immunoassay kit (R & D Systems). GM-CSF was detected in supernatant fluids of MOLT-14 cells treated with APP using the GM-CSF ELISAMax kit (BioLegend, San Diego, CA, USA). All experiments were performed in triplicate according to the respective manufacturer's instructions. Data are presented as average ± standard deviation.

Peritonitis Studies

BALB/c or $CXCR2^{-/-}$ (gift of A. Harmsen, Montana State University) mice were injected in the peritoneum with APP (1mg in PBS, optimal dose and timing were empirically determined) or different doses of purified procyanidins, and after 3h blood was collected into blood collection Microvette® 200 tubes (Sarstedt, Newton, NC, USA) with sera separated by centrifugation at 12 000 *x g* for 10m. Red blood cells were lysed in ACK buffer (0.15M $NH_4Cl$, 1mM $KHCO_3$, 0.1mM EDTA), washed in Hank's Buffered Saline Solution (HBSS, Mediatech), and leukocytes were analyzed by flow

cytometry as described below. After euthanasia, peritoneal cavities were washed with 300μL HBSS (injected and retrieved) followed by a 5mL wash with HBSS. Retrieved HBSS from the 300μL wash was used for CXCL1/KC ELISAs. Cells from the 5mL wash were collected by centrifugation at 500x*g* for 5m and similarly subjected to flow cytometric analysis. Blood and peritoneal cells were stained with directly conjugated RB6-8C5-FITC (anti-granulocyte receptor-1; Bright on all neutrophils), anti-CD45.2-APC (all leukocytes; disregards RBCs) and anti-CD11b-PE (bright on neutrophils) and read on a FACSCalibur equipped with an HTS loader (BD Biosciences, San Jose, CA, USA). Viable leukocytes were gated based on FSC/SSC and positive CD45 staining. Of the viable leukocytes, the percentage of neutrophils (RB6-8C5$^{bright}$/CD11b$^{bright}$ and distinctive light scatter profile) was determined. All animal procedures were performed in accordance with National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee of Montana State University (Bozeman, MT).

Western Immunoblots

MOLT-14 cells were treated with PBS or APP for 30m, 60m, and 4h at the concentrations described above. At the indicated time-points, cells were transferred to tubes and collected by centrifugation for 5m at 500x*g*. Supernatant fluids were removed and discarded and pellets were suspended in buffer containing 0.15M NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, and 10mM Tris-HCl pH 7.2, and were incubated on ice for 30m. Samples were boiled for 5m prior to resolution on a 10% SDS-

PAGE gel. Proteins in the gels were transferred to nitrocellulose then blocked in 10% Blotto (10% w/v nonfat dry milk in PBS) for 1h at room temperature. Blots were incubated with the following antibodies diluted 1:1 000 in 0.5% Blotto at room temperature overnight: anti-phospho ERK 1/2 MAPK (Thr202/Tyr204)(Cell Signaling, Danvers, MA, USA), anti-phospho p38 MAPK (Thr180/Tyr182) (Cell Signaling), anti-phospho JNK1/JNK2 MAPK (Thr183/Tyr185) (Abcam, Cambridge, MA, USA), anti-ERK1/2 MAPK (Cell Signaling), anti-p38 MAPK (Cell Signaling), or anti-JNK1/JNK2 MAPK (also known as SAPK/JNK) (Cell Signaling). Blots were washed three times with 0.5% Blotto and incubated with HRP-goat anti-rabbit secondary antibody at 1:5 000 in 0.5% blotto for 2h at room temperature. Blots were washed and developed with ECL reagent (GE Healthcare, Piscataway, NJ, USA) and exposed to film for autoradiography.

## Results

### APP Induces Robust Changes in Global Transcript Expression in Primary Bovine γδ T Cells.

To broaden our understanding of the transcriptome profile induced in γδ T cells treated with APP beyond a few select cytokines, a microarray experiment was performed using primary sorted bovine γδ T cells. We used the advantage of the bovine system for these experiments as large numbers of γδ T cells could be purified to near homogeneity from neonatal animals.[288] Importantly, the APP preparation used in these studies has been documented to contain 95% polyphenolic compounds, the majority of which are procyanidins, with undetectable or very little endotoxin[1]. Cells were treated with buffer

carrier or APP for 4h and then RNA was extracted and used to probe an Affymetrix Bovine Genechip array as described.[287] APP treatment of γδ T cells significantly increased transcript levels for approximately 2 000 genes at least two-fold while there were few genes significantly down-regulated in the APP- versus PBS-treated γδ T cells (see GEO series number GSE13321). The most robust response was observed for the *Gmcsf* transcript, which was induced approximately 85-fold over PBS-treated cells (Table 3.1). Transcripts for activation markers such as *Cd69* and *Il2ra* were also induced over PBS-treated control cells. Other transcripts with increased expression included *Vegf*, an endothelial growth factor, and NK cell molecules including the killer cell lectin-like receptor 1, but the significance of their expression was not further analyzed. Interestingly, overt inflammation markers such as IFNγ were not detected in these experiments. However, other inflammatory molecules such as *Tnfa* and *Il8* were induced 5.8-fold and 3.1-fold, respectively. This pattern of gene expression was consistent with γδ T cell priming[1,3] and other work in our laboratory[117] where procyanidins induce a unique inflammatory profile in γδ T cells that does not include expression of IFNγ.

Table 3.1 Immune-Relevant Genes with Greater than 3-fold Induction after Treatment of Bovine γδ T Cells with APP

| APP fold change over PBS | Gene Description |
|---|---|
| 85.2 | GM-CSF (colony stimulating factor 2) |
| 21.3 | Vascular endothelial junction-associated molecule (VE-JAM) |
| 19.5 | IL-2 |
| 19.1 | Killer cell lectin-like receptor F1 |
| 11.8 | Chemokine (C-C motif) ligand 3-like 1 |
| 9.2 | MIP-1β |
| 8.8 | IL-4R |
| 8.8 | CD83 (B-cell activation protein) |
| 7.3 | VEGF (vascular endothelial growth factor) |
| 7.2 | TGFβ1 (transforming growth factor beta 1) |
| 7.0 | IL-13 |
| 6.9 | CD69 (early T-cell activation antigen) |
| 6.7 | CCR5 (chemokine receptor 5) |
| 6.1 | Chemokine (C-X-C motif) ligand 2 |
| 6.0 | IL-1Rα |
| 6.0 | IL-2Rα |
| 5.8 | TNFα (tumor necrosis factor, alpha) |
| 5.8 | Chemokine (C-X-C motif) ligand 16 |
| 5.7 | Chemokine (C-C motif) ligand 20 |
| 5.4 | MHC class I heavy chain |
| 5.2 | Chemokine (C-X-C motif) ligand 17 |
| 4.1 | CD82 antigen |
| 4.1 | Chemokine (C motif) ligand 1 |
| 3.9 | IL-17 |
| 3.7 | ODC 1 (ornithine decarboxylase 1) |
| 3.1 | IL-8 |

## APP Procyanidins Prime Mouse γδ T Cells *in vitro* and Alter the Mouse Cytokine Profile *in vivo*, Resulting in Neutrophil Influx to the Blood and Peritoneum

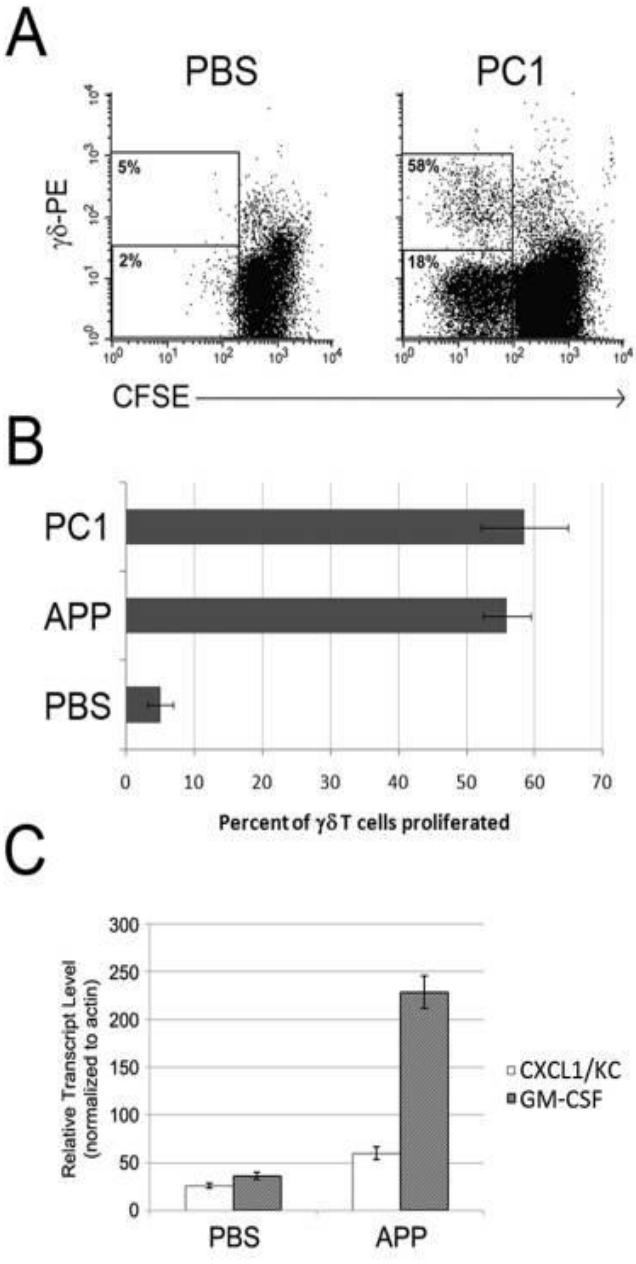
An increased γδ T cell population in the intestinal mucosa of mice[238] and rats[239] is observed after consumption of APP, suggesting that a murine response occurs similar to that described for bovine and human γδ T cells.[1-3,117] As such, we wanted to determine if

mouse γδ T cells similarly respond to APP *in vitro*. To test for a mouse response to oligomeric procyanidins, we used a commercially-available, highly-purified oligomeric trimer (PC1). Priming was quantified by the number of γδ T cells that proliferated following APP and PC1 treatment, compared to the un-primed (buffer control) culture. Procyanidin treatment induced γδ T cell proliferation in response to IL-2 (Figure 3.1A[BALB/c] and B [C57BL/6]), indicating APP and purified procyanidins acted as γδ T cell priming agents in two common inbred mouse strains. Similar to previous experiments with bovine and human cells, a small percentage of non-γδ T cell lymphocytes proliferated. In human cultures, the non-γδ T cells responding to procyanidin are predominantly NK cells, however this procyanidin-responsive population was not characterized in mice.[1]

Next we tested for the ability of mouse cells to increase expression of the prototypical priming transcripts encoding *Gmcsf* and *Il8*. Spleen cell preparations from BALB/c mice were treated with buffer/carrier or APP for 4h and *Cxcl1/Kc* (*IL8* homolog), as well as *Gmcsf* transcripts, were measured by quantitative RT-PCR. As shown in Figure 3.1C, both cytokine transcripts were readily detected following APP treatment.[2] Since *IL8* and *GMCSF* transcripts are expressed in procyanidin-treated cells from human (unpublished data) and bovine (Table 3.1), and were confirmed in mouse, we chose to focus the remainder of our studies on these two cytokines.

We next performed *in vivo* experiments to assess a functional role for procyanidin-induced CXCL1/KC and GM-CSF. Since GM-CSF and CXCL1/KC induce the

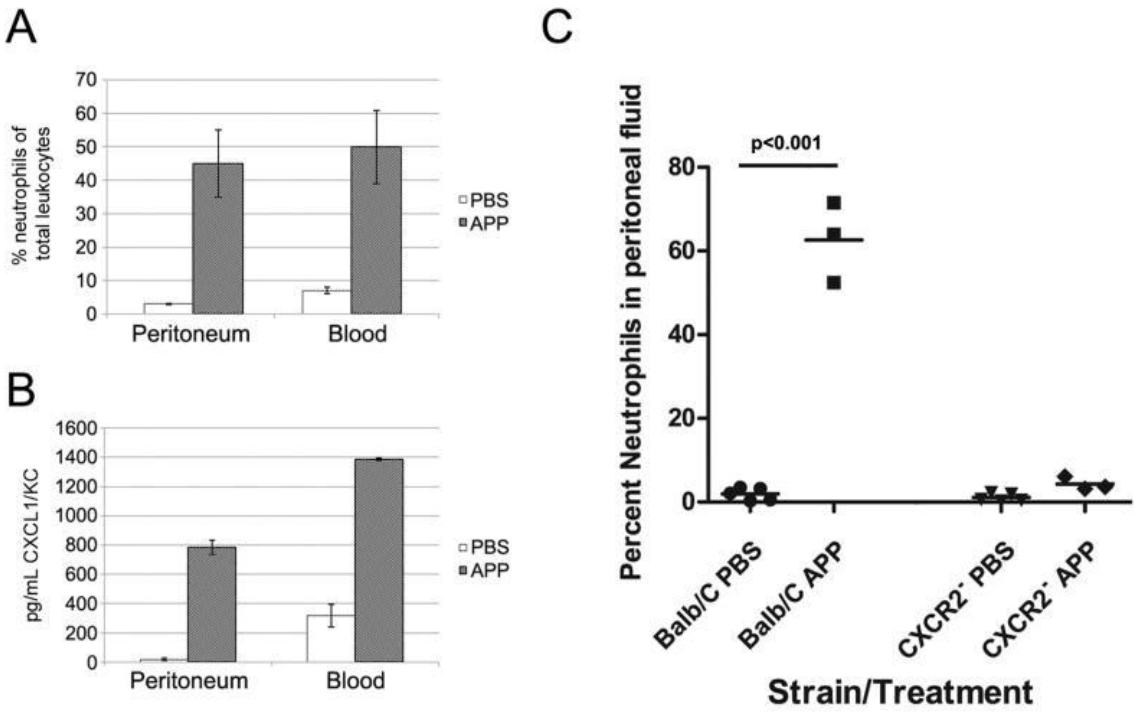
Figure 3.1 APP and/or Purified Procyanidin (PC1) Prime Mouse γδ T Cells to Proliferate in Response to IL-2 and Induce *Cxcl1/Kc* and *Gmcsf* Transcript Expression in Murine Splenocytes




A) CFSE-labeled BALB/c splenocytes were cultured in X-VIVO-15 medium with PBS or PC1 (15μg/mL) for 24h. Culture supernatant fluid was then replaced with fresh medium containing 1ng/mL rhIL-2 and cultured for an additional 72h. Cells were then stained for γδ TCR (GL3) and analyzed by flow cytometry. A representative example of a two-color FACS plot from one of three experiment repeats is shown. Values represent the percent proliferated of either the γδ T cell (top region) or non-γδ T cell (bottom region) populations. Percent proliferation was measured by dividing the number CFSE-dim cells (gated) from the number of total cells in the respective population. B) Experiments performed as described in (A) were also completed using spleen cells from C57BL/6 mice, and responses to APP and PC1 compared. Data represent the average +/− SD from 3 experiments ($P<0.001$ for both APP and PC1 vs. buffer control). C) Spleen cells isolated from BALB/c mice were treated with APP or PBS in X-VIVO for 4h. RNA was extracted from each sample and subjected to quantitative RT-PCR for actin, *Gmcsf*, and *Cxcl1/Kc*. Results represent mean +/− SD from triplicate values.

development of a peritonitis response,[289,290] we chose this model to measure APP responses *in vivo*. Neutrophil influx was measured as the ratio of neutrophils (RB6-8C5$^{bright}$, CD11b$^{bright}$) to total leukocytes (CD45.2$^{+}$). Substantial neutrophil influxes were observed in both the blood (neutrophilia) and the peritoneum (recruitment) of mice injected with APP (Figure 3.2A). Administration of purified procyanidins likewise induced neutrophilia; as little as 50 μg PC1 induced significant neutrophil accumulation (data not shown). As shown in Figure 3.2B, approximately 785 pg/mL CXCL1/KC was detected in the IP fluid wash, and 1,400 pg/mL was detected in the blood of mice injected with APP, confirming that the neutrophil response observed in APP-injected mice correlates with the expression of CXCL1/KC. Likewise, neutrophil recruitment to the peritoneum was observed when CXCR2$^{-/-}$ mice, which lack the major CXCL1/KC receptor, were used in the same procedure, thereby confirming a role for CXCL1/KC in this process (Figure 3.2C). GM-CSF was not detected by ELISA in the same sample fluids at the 4h time point (unpublished data). It is possible that GM-CSF could be detected at time points different than those tested, but it is also possible that the ELISA method used here was not sensitive enough for this model. These gene and protein expression data and *in vivo* responses suggested that GM-CSF and CXCL1/KC play a key role in affecting the procyanidin response *in vivo*, and thus these transcripts were further analyzed.

Figure 3.2 APP Induces CXCL1/KC in the Peritoneum and Blood of Mice Resulting in Neutrophilia.




A) BALB/c mice were injected IP with 1mg of APP prepared in PBS, or with PBS alone. After 4h, blood and IP fluid were harvested from each animal and the cells in each sample were subjected to FACS analysis. Data are presented as the mean percentage (+/−SD from 3 mice) of neutrophils out of the total number of viable leukocytes in the peritoneum and are representative of a minimum of three independent experiments. **B)** BALB/c mice were injected IP with 1mg of APP prepared in PBS, or with PBS alone. After 4h, blood and IP fluid were harvested from each animal and were subjected to ELISA for CXCL1/KC. Data are the mean (+/− SD) concentration (pg/mL) of CXCL1/KC in each sample. C) Experiments were done as described for (A) and comparisons in the APP response in the peritoneum of BALB/c mice and mice lacking CXCR2 were made. p-value was determined using Student's unpaired T test.

## *GMCSF* and *IL8* Transcripts are Stabilized in Human γδ T Cells During Treatment with APP

After validating that procyanidin effects in the mouse were similar to previous reports for other species[1] and demonstrating an *in vivo* relevance for procyanidins, we next sought to ascertain the mechanism for the rapid response to secondary stimuli after priming with APP.[1] In an effort to control for variability in primary cell cultures and the limited number of γδ T cells available in such cultures, we selected a cell line. Several cell lines were tested for procyanidin responsiveness including: THP1, Monomac-1, Jurkat, Lucy, PEER, and MOLT-14 cells (data not shown). Of those tested, only MOLT-14 cells, a γδ $TCR^+$ human leukemia cell line, up-regulated *GMCSF* and *IL8* transcripts as well as generated GM-CSF in response to procyanidin culture (Supplemental Figure 3.1 [Available online under NIH public manuscripts, PMC3136559] and data not shown). Thus, using the MOLT-14 cell line, we designed experiments to begin to define the mechanism by which *GMCSF* and *IL8* transcript and protein are induced in cells treated with APP.

Based on the rapid responses we previously observed in primed γδ T cells, we hypothesized that these transcripts contain structural features enabling this rapid induction.[1] Upon analyzing the transcripts for similar structures, we observed that *GMCSF* and *IL8* contain similar AU-rich elements (AUREs) that function to modulate transcript degradation rates.[291] Therefore, the stability of these transcripts was analyzed following treatment with APP. MOLT-14 cells were treated with APP or TNFα for 4h, and then with actinomycin D to prevent *de novo* transcription. RNA was harvested at

time 0 (collected immediately before actinomycin-D treatment), 30m, 90m, and 4h post-actinomycin D treatment, and samples were analyzed by quantitative RT-PCR using 18S to determine relative expression. Since MOLT-14 cells constitutively express very low *GMCSF* and *IL8* transcripts (data not shown), treatment with TNFα was used as a negative control as it induces expression of *GMCSF* transcript but does not stabilize it.[292] *GMCSF* transcript at the time of collection (t=0) in TNFα-treated cells was approximately 11.8-fold less than in APP-treated cells, and 2.1-fold greater than in PC1-treated cells. Initial *IL8* transcript was similar in APP- and TNFα- treated cells (1.4-fold greater than TNFα-induced transcript). In APP-treated cells, the *GMCSF* transcript decreased to 49% of the initial amount 30m after actinomycin D-induced transcription blockade, and remained at 48% and 17% of the initial amount after 90m and 4h, respectively (Figure 3.3A). By comparison, the transcript was degraded quickly in TNFα-treated cells; *GMCSF* transcripts decreased to 20% of the initial amount at 30m, and were nearly equivalent to PBS-treated cells at both 90m and 4h following TNFα-induced induction. These results indicate that APP- but not TNFα-induced *GMCSF* transcripts were likely stabilized in MOLT-14 cells.

We also tested whether the purified PC1 trimer also influenced the stability of the *GMCSF* transcript in MOLT-14 cells. As shown in Figure 3.3B, PC1 both induced and stabilized the *GMCSF* transcript. Though the induced expression of *GMCSF* by PC1 was not as robust as that induced by APP (t=0 was 22-fold less than with APP), treatment with PC1 resulted in a stability pattern similar to that for APP. These data confirm that

defined procyanidin species, such as the PC1 trimer, induce transcript stability in γδ T cells.

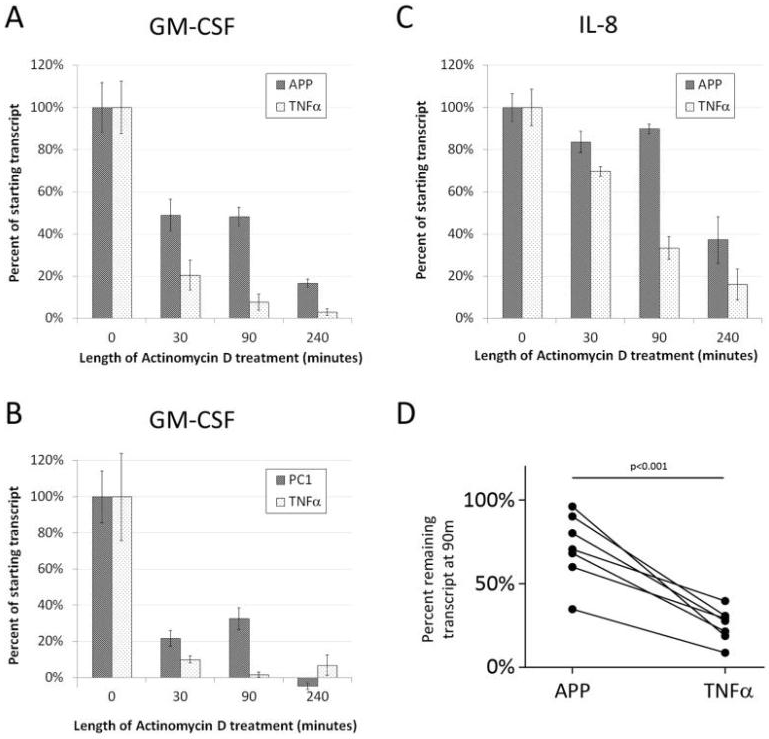
We then performed similar experiments using the IL-8 transcript. As shown in Figure 3.3C, the *IL8* transcript was similarly regulated by APP and TNFα, although induced *IL8* levels were less when compared to *GMCSF* (4-fold increase versus background vs. 919.7-fold increase for *GMCSF*). TNFα-induced *IL8* transcript expression was not stabilized and was equivalent to cells treated with PBS by 90m. In comparison, treatment with APP resulted in *IL8* stabilization, which remained significantly greater than background (PBS), even 4h later.

Previous reports demonstrate predicted half-lives for *GMCSF* and *IL8* to be ~30m[293] and ~20m[294] respectively. Therefore, we predicted un-stabilized transcripts would be greatly diminished at 90m (3 half-lives for *GMCSF* and 4.5 half-lives for *IL8*), and in fact this was observed in these experiments, with the greatest difference in transcript retention between APP- and TNFα-treated cells being observed 90m after transcription was inhibited via actinomycin D. Since the greatest change in transcript stability was observed 90m after transcript blockade, we chose this time point when validating our methods.

We performed three additional experiments to address potential artifact in these transcript stability experiments. First, to address the potential that the observed transcript stabilization was an artifact of the MOLT14 cell line, we tested primary cultures for increased stabilization in APP-treated cultures. Bovine PBMC cultures were selected because they contain a higher percentage of responding cells than human or mouse

PBMCs. As shown in Figure 3.3D, primary PBMC cultures treated with APP had more stable *GMCSF* transcripts than TNFα-treated cultures. This demonstrated that the transcript stabilization in MOLT14 cells was not an artifact of the cell line. Secondly, to control for artifact due to actinomycin D in the t=30, 90, and 240, but not t=0 RNA preparations, we added actinomycin D to all samples, including t=0, prior to collection. Similar to previous experiments (Figures 3.3A and 3.3C), we observed rapid degradation in TNFα-, but not APP-treated cultures (Supplemental Figure 3.2A; online: PMC3136559). Finally, since procyanidins bind with high avidity to proteins and other compounds,[2] we questioned whether the observed transcripts could be a result of APP-induced actinomycin D precipitation and thereby incomplete blockade of transcription. To address this potential artifact, we tested whether removal of APP at the point of actinomycin D treatment had an effect on transcript maintenance. To this end, we treated bovine PBMCs with APP for 4h, washed twice with medium to remove free procyanidin, and added fresh medium containing actinomycin D with either APP or PBS. Ninety minutes after the fresh media were added, RNA was collected from the cells and *GMCSF* transcripts were assayed by RT-PCR. As expected, due to the outbred nature of calves, as well as varying ages of individuals, there were differences in *GMCSF* transcript levels from animal to animal. However, there was no difference in transcript levels when actinomycin D was incubated in the presence or absence of APP (Supplemental Figure 3.2B; online: PMC3136559), thereby indicating APP did not affect actinomycin D activity.

Figure 3.3 APP Stabilizes *GMCSF* and *IL8* Transcripts in MOLT-14 Cells




A–C) MOLT-14 cells were treated with PBS, APP, TNFα, or PC1 in cRPMI for 4h and then collected (t=0) or treated with actinomycin D for the length of time indicated in the figure. RNA was extracted from each sample and subjected to quantitative RT-PCR for *18S*, *GMCSF* (panels A and B), or *IL8* (panel C). Data represent the percent remaining transcript as measured by the mean relative expression of triplicate values minus background (PBS-treated cells 4h after actinomycin D treatment) +/− SD. Data are representative of three independent experiments. PC1 experiments (panel B) were performed as in A except separately and with different TNFα-and PBS-treated controls. D) Primary bovine PBLs from calves were cultured with APP (50μg/mL) or TNFα (50ng/mL) for 4h. The cultures were then treated with actinomycin D (ActD) by spiking directly into the culture (n=4) or by replacing the media with fresh medium containing actinomycin D (n=3). Cultures were harvested immediately after actinomycin D treatment and again 90m later. RT-PCR was performed to determine *GMCSF* transcript relative to *18S*. The relative percentage of transcript remaining for TNFα and APP at 90m vs. 0m is shown. Error bars represent SD. The p-value was determined using a one-tailed Wilcoxon's signed rank test. Data from APP-treated PBMCs with medium replacement were also used as part of the data set in Supplemental Figure 3.2B. Without these data, the p-value is significant to $p<0.01$.

## The ERK1/2 Pathway is Activated in MOLT-14 Cells Treated with APP

We next performed experiments to identify the pathway(s) that could be responsible for the transcript stabilization observed in cells treated with procyanidins. We began by investigating the possible activation of a MAPK pathway, as signaling through these modules is important in transcript stability.[295-298] MOLT-14 cells were treated with PBS or APP for 30m, 60m, or 4h, whereupon cell lysates were prepared and subjected to SDS-PAGE and Western immunoblot using antibodies specific for the phosphorylated MAP Kinases, ERK1/2 (p44/p42), p38, and JNK. Blots were subsequently stripped and re-probed using antibodies specific for the unphosphorylated form of the corresponding protein to verify equal loading between lanes. As shown in Figure 3.4A, the ERK1/2 molecules were phosphorylated to high levels in cells treated with APP, but not in those treated with PBS alone. p38 MAPK was not phosphorylated in response to APP, whereas APP did result in the phosphorylation of JNK1 and JNK2 (Supplemental Figure 3.3; online PMC3136559), suggesting activation of the JNK/SAPK MAPK pathway.

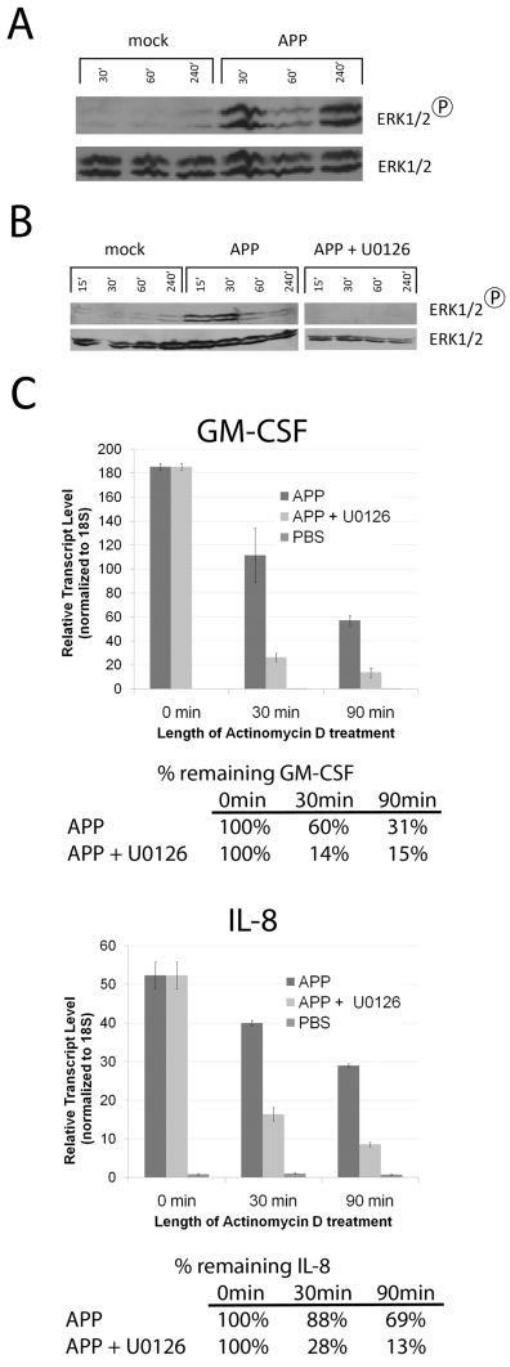
## The ERK1/2 MAPK Pathway is Required for *GMCSF* and *IL8* Transcript Stabilization in MOLT-14 Cells Treated with APP

Since the ERK and p38 pathways have been reported to stabilize AURE-containing transcripts, and the p38 pathway was shown to not be activated during procyanidin culture, we next tested whether the ERK1/2 MAPK pathway plays a role in the increased stability of the *GMCSF* and *IL8* transcripts in APP-treated MOLT-14 cells. To this end, we employed an ERK1/2 inhibitor in stability experiments. U0126 inhibits MEK1/2, the

upstream kinase of ERK1/2, and thus inhibits any signaling events downstream of ERK1/2 phosphorylation.[299] In these experiments, MOLT-14 cells were treated with APP for 2h to induce gene transcription, and then with 20 μM U0126 for an additional 2h. This treatment condition was determined to inhibit ERK1/2 phosphorylation in MOLT-14 cells by Western immunoblot (Figure 3.4B). Cultures stimulated for 4h as above with PBS, APP, or APP/U0126 were then treated with actinomycin D, and RNA samples were harvested at 0m, 30m and 90m. As shown in Figure 3.4C, treatment with APP induced stability of the *GMCSF* transcript as previously seen, but treatment with U0126 prior to actinomycin D treatment abolished this effect (presence of the transcript was reduced to 14% of the initial total by 30m and to 7% by 90m in the presence of U0126, compared to 60% and 31%, respectively, when cells were treated with APP alone). A similar observation was made for the *IL8* transcript in cells pre-treated with U0126, where its presence was reduced to 54% and 39% of the initial concentration after 30m and 90m of actinomycin D exposure, respectively. In contrast, APP-treated cells not treated with U0126 retained 89% and 73% of *IL8* transcript after 30m and 90m, respectively (Figure 3.4C). These data suggest that the ERK1/2 pathway is at least partially responsible for the induced stability of these transcripts.

We next tested the ability of U0126 to regulate the decay rate of *JUN*, a quickly degraded (half-life of ~20m)[300] transcript containing a class III AURE (as opposed to the class II AURE from *IL8* and *GMCSF*). MOLT-14 cells were treated with APP for 2h, then with the U0126 inhibitor for 2h, followed by treatment with actinomycin D. RNA was harvested at 0m, 30m, and 90m post-actinomycin D treatment, and RT-PCR was

Figure 3.4 APP Activates the ERK1/2 Pathway, which is required for *GMCSF* and *IL8* Stabilization



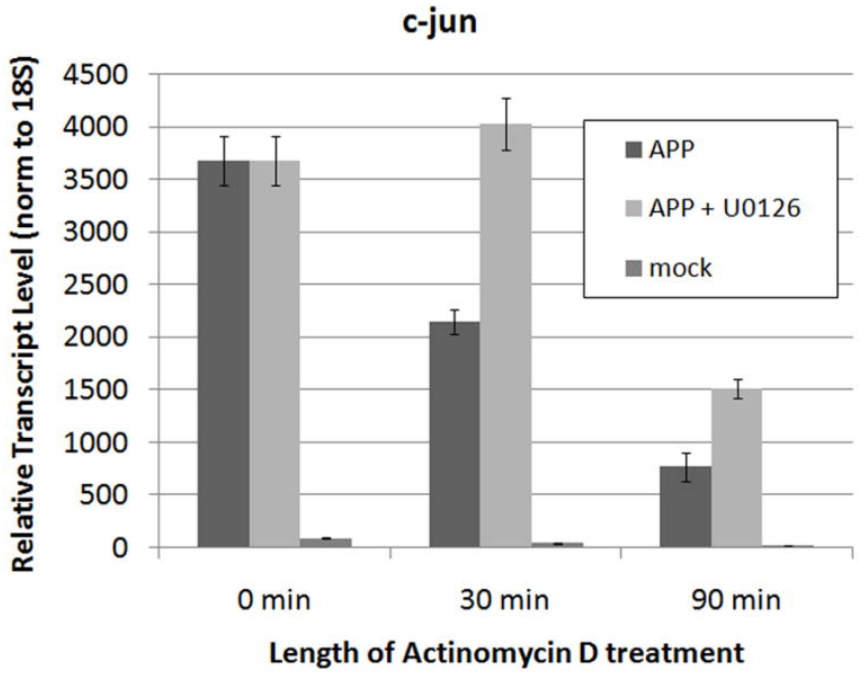

A: MOLT-14 cells were treated with PBS or APP in cRPMI for the time indicated in the figure. Cell lysates were prepared and subjected to Western immunoblot for phospho-ERK1/2 or total ERK1/2. Data are representative of three independent experiments. B: Cells were treated with APP, APP and 20μM U0126, or mock and harvested at 15, 30, 60, and 240m. Cells were then lysed and analyzed for phospho-ERK1/2 or ERK1/2 via western blot. C: MOLT-14 cells were treated with PBS or APP in cRPMI for 2h, followed by treatment with U0126 for 2h, and then with actinomycin D for the time indicated in the figure. RNA was extracted from each sample and subjected to quantitative RT-PCR for *18S*, *GMCSF*, and *IL8*. Data represent mean of triplicate values +/− SD and are presented as the normalized amount of each transcript to 18S. The percentage of each transcript that remained following treatment was calculated after subtracting background (PBS t=90m). Data are representative of three independent experiments

performed as previously described. As shown in Figure 3.5, c-Jun transcript expression was induced in cells treated with APP at time 0, as expected. The presence of the *JUN* transcript decreased over time in cells treated with APP. When cells were pre-treated with the U0126 inhibitor prior to actinomycin D treatment, the decay rate of the *JUN* transcript was not accelerated. In fact, treatment with the inhibitor potentially stimulated transcript production despite the presence of actinomycin D. Importantly, this observation is distinct from what was observed for both the *GMCSF* and *IL8* transcripts, and suggests that APP-induced ERK1/2 activation is specific for these class II AURE-containing transcripts. Together, these data suggest that transcript stabilization may be a mechanism for γδ T cell priming in response to procyanidins.

## Discussion

This report begins to define the mechanisms that are involved in our previous reports of procyanidin-induced γδ T cell priming. Many transcripts associated with inflammation, including *IL8* and *GMCSF*, were found to be increased in expression upon procyanidin stimulation. However, the effect on γδ T cells is unlike TCR stimulation in that IFNγ transcript levels are unchanged.[117] We show *in vivo* relevance for the procyanidin-induced cytokine response with the observation that injection of APP results in a rapid neutrophil influx into both the peritoneum and blood. Furthermore, these studies show that *GMCSF* and *IL8* transcripts are stabilized in cells treated with procyanidins and this likely occurs via ERK1/2 MAPK signaling. Together, these data describe a mechanism where cytokines involved in the generation and recruitment of

Figure 3.5 The ERK1/2 Pathway Inhibitor U0126 does not Prevent *JUN* Transcript Stabilization in MOLT-14 Cells Treated with APP




% remaining c-jun

| | 0min | 30min | 90min |
|---|---|---|---|
| APP | 100% | 58% | 21% |
| APP + U0126 | 100% | 109% | 41% |

MOLT-14 cells were treated with PBS or APP in cRPMI for 2h, followed by treatment with U0126 for 2h, and then with actinomycin D for the time indicated in the figure. RNA was extracted from each sample and subjected to quantitative RT-PCR for *18S* and *JUN*. Data represent mean of triplicate values +/−SD and are presented as the normalized amount of each transcript to 18S. The percentage of each transcript that remained following treatment was calculated after subtracting background (PBS t=90min). Data are representative of three independent experiments.

neutrophils are induced and stabilized by APP procyanidins. The microarray analyses performed on γδ T cells indicate that these responses are similar to PAMP-induced γδ T cell priming.[1] These PAMP responses are characterized as mild inflammatory responses, leading to increased responses upon secondary stimulation. This may prove a useful alternative to PAMP agonists currently being tested in clinical applications as these candidates have undesired side-effects.

These microarray results also confirmed our previous reports[117] that *Gmcsf* is dramatically up-regulated during APP treatment (85.2-fold increase). When tested in mice, procyanidins, including purified PC1, elicited similar responses to those defined in humans and cattle. Since oral delivery increases γδ T cell populations in the gut,[238] our confirmation of a mouse response is not surprising, however characterization of a phenotype in these different tissues demonstrates that the effect of procyanidins is not limited to the intestinal mucosa. Furthermore, using this peritonitis model, we were also able to confirm the importance of the secreted cytokine CXCL1/KC (functional equivalent to IL-8).

Using CXCL1/KC (IL-8) and GM-CSF as key cytokines in the procyanidin response, we looked for a mechanism that could explain the γδ T cell priming response. Both of these transcripts contain an AURE in their 3' UTR,[301,302] which suggested that the presence of these elements play a role in the reduced degradation rate of these mRNAs in APP-treated MOLT-14 cells. These studies demonstrated that both the *GMCSF* and *IL8* transcripts were stabilized in cells treated with APP. AURE-containing transcript degradation is often regulated and associated with p38 and/or ERK1/2 MAPK activation.

These results indicate that ERK1/2 but not p38 activity is important for the increased half-life of the *GMCSF* and *IL8* transcripts in APP-treated MOLT-14 cells. This finding is uncommon, but has previously been described in the context of transcript stability.[303] Conversely, the p38 MAPK pathway is commonly implicated in stabilizing transcripts in other systems,[294,296,297,304] but APP-mediated transcript stability appears independent of the p38 kinase, since its phosphorylation was not observed (data not shown). Perhaps the fact that the p38 MAPK pathway is not activated in APP-treated cells and is instead regulated by the ERK1/2 MAPK accounts for the unique inflammatory phenotype we detect. This conclusion is supported by prior studies that observe p38 MAPK activation in response to cellular stress, while the ERK1/2 MAPK module primarily responds to mitogenic stimuli.[305-308] Previously, we found that APP enhanced γδ T cell proliferation in response to either IL-2 or IL-15.[1] Selective activation of the ERK1/2 MAPK pathway is consistent with these observations. Likewise, activation of the ERK1/2 MAPK pathway results in an anti-inflammatory cellular environment[309] and could explain why, even in the face of robust induction of some inflammatory cytokines, expression of the prototypic inflammatory cytokines of γδ T cells, such as IFNγ, is not seen. Investigation into the role of the JNK MAPK pathway in APP-treated MOLT-14 cells will also shed light on APP-mediated mechanisms, since the JNK module, like p38, signals in response to events such as inflammation and apoptosis.[310-312]

Although it is not clear how MAP kinases regulate AURE degradation, studies have revealed that they can both phosphorylate and regulate the activity of at least some AURE-binding proteins. There are many immunologically-relevant AURE-BPs that can

affect transcript stability and can have opposing roles in transcript degradation based on the presence, absence, or phosphorylation state these proteins. Commonly-described AURE-BPs include the following: TTP, BRF1, BRF2, Roquin, and CUGBP1, which promote transcript degradation, HuR, NF90, YB-1, and AUF2, which prevent degradation, and AUF1 and KSRP, which can either induce or prevent transcript degradation. Many of these AURE-BPs have been shown to regulate *GMCSF* and/or *IL8* (*Cxcl1/Kc*) transcript stability including AUBF,[313] YB-1,[314] TTP,[302,315] HuR,[316] and KSRP.[302] Since the *GMCSF*, *IL8*, and *Cxcl1/Kc* transcripts contain Class II AUREs,[301,302,317,318] it is possible that regulation of their decay is related to the activity of a common protein. Further experiments are underway to fully understand MAP kinase activity and AURE-BP interactions during procyanidin treatment.

These studies shed some light on the apparent dichotomy existing with respect to the function of procyanidin molecules. Procyanidins are proposed for the treatment of inflammatory diseases,[319] as well as in the clearance of infection,[320,321] indicating that they have both anti-inflammatory and pro-inflammatory properties. For example, some procyanidins can inhibit NFκB activity[228,257,322-325] and play a role in inhibiting the development of food allergies[238] and allergic rhinitis.[326] Likewise, procyanidins from grape seeds have anti-tumor activity.[327] In contrast, procyanidins from cocoa have a pro-inflammatory effect on PBMCs by inducing cytokine release.[236] Our data point to an inflammatory phenotype for γδ T cells treated with APP, as evidenced by the nature of the induced cytokines such as GM-CSF and IL-8. However, it is clearly a restricted phenotype in that overt activation markers, such as IFNγ, are not induced, and activation

of the ERK1/2 and JNK1/2 MAPK but not p38 pathways are detected. Perhaps this limited inflammatory response contributes to downstream regulatory responses that ultimately dampen or control expression of other inflammatory molecules. Alternatively, it seems other AURE-containing transcripts could be stabilized during procyanidin treatment. If this were to occur, it would stand to reason that the production of these cytokines, should the cell be producing them, would similarly increase. This could lead to an improvement in the response, should it be inflammatory or suppressive. Clearly, these questions remain to be answered, however the demonstration that mice respond to procyanidins will provide an accessible model system to test these hypotheses under both inflammatory and infectious conditions.

Collectively, the data reported herein divulge a mechanism by which APP rapidly and robustly induces a unique response *in vitro* and *in vivo*. Importantly, we have begun to define a cross-species mechanism by which γδ T cells, thought to be a bridge between the innate and adaptive immune systems, are primed in response to APP. Our model predicts that this priming response could enhance either pro-inflammatory or immunoregulatory activities of γδ T cells, which would be dictated by the nature of a secondary stimulus. Although we are focused on γδ T cells, these mechanisms could also be involved in procyanidin effects on other lymphocytes, such as NK cells and subsets of αβ T cells that also respond, as shown in our earlier study[1]. Further defining these mechanisms and their impact *in vivo* will provide insight into optimizing the use of these types of compounds to treat inflammatory diseases[319] as well as enhancing the clearance of various infections.[320,321]

Acknowledgements

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# CHAPTER FOUR

## OLIGOMERIC PROCYANIDIN-MEDIATED TRANSCRIPT STABILITY IS DEPENDENT UPON SYK AND ERK KINASES

### Contribution of Author and Co-Author

Manuscript in Chapter Four

Author: Jeff Holderness

Contributions: Designed and performed experiments, prepared the manuscript.

Co-author: Mark A. Jutila

Contributions: Designed experiments and obtained funding.

Manuscript Information Page

- Jeff Holderness and Mark A. Jutila
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  - ___ Accepted by a peer-reviewed journal
  - ___ Published in a peer-reviewed journal

## Abstract

We recently demonstrated that γδ T cells, an innate immune cell found in abundance in the tissues and responsible for regulating many immune responses, can become primed in response to procyanidins. This response includes up-regulation of cytokine receptors, production of select cytokines, and stabilization of gene transcripts for *GMCSF* and *IL-8/Cxcl1*. The induced stability of gene transcripts is mediated by ERK. To further elucidate the mechanisms for this phenomenon, we generated a γδ TCR-expressing reporter line containing GFP with the 3'UTR from *GMCSF*. In this line, GFP transcript was stabilized leading to increased transcript and protein expression upon OPC treatment but not by other immunologically-relevant polyphenols including the monomeric procyanidins, the 67kDa Laminin Receptor ligand, EGCG, or the Nrf2 agonist, resveratrol. To further elucidate the mechanisms of this process, we screened kinase inhibitors in an effort to characterize the procyanidin-mediated pathway of transcript stability. In addition to ERK, we identified syk as involved in this stabilizing mechanism.


## Introduction

It is becoming increasingly clear that the regulation of innate immune cells by plant products is not mediated only by their ability to quench oxidants, but that receptor mediated responses are required. The characterization of many polyphenolic compounds capable of inducing Nrf2 antioxidant pathway explains the host responses of some

polyphenols, but not others. Recently, we described the ability of OPCs to activate γδ T cells. The γδ T cell is an innate immune cell with effector functions that can modulate inflammatory responses as well as suppress inflammation and promote wound repair. This multifunctional cell is particularly prevalent at sites proximal to the surface, where it maintains barrier integrity. Thus, there are potential applications a γδ T cell agonist in innate immune activation and protection.

Activation of the γδ T cell population by extracts from unripe apple peels or the bark of Cat's Claw was originally tracked to the OPC fractions. The phenotype induced by OPCs leads to a priming response that is similar to priming by PAMP engagement of the toll-like receptors. These responses are associated with increased expression of activation receptors and MIP-1α production, but not overt inflammation or proliferative responses. This priming response occurs with dimeric and larger OPCs, but not their monomeric subunits. Recently, we also identified OPC-induced changes to the transcriptome of γδ T cells. During OPC treatment, transcripts for *GMCSF* and *IL8* become stabilized. These transcripts are part of a family of sensitive transcripts that are rapidly degraded by means of an AU-rich element (AURE) in their 3'UTR. Normally, *GMCSF* transcript has a half-life of approximately 15m. We found that treatment with OPCs dramatically increases this, often to over a 90m half-life. The transcript for *JUN* is not affected, indicating this response was selective for a subset of AURE-containing genes.

To determine if the changes to *GMCSF* transcript degradation induced by OPCs were, in fact, mediated by the 3'UTR, we produced a GFP-expressing reporter containing the 3'UTR from *GMCSF*. As expected, *GFP-3'UTR-GMCSF* transcript was stabilized during OPC treatment, resulting in an increase cellular accumulation of GFP. Using this cell line, we tested various polyphenol preparations, signaling pathway inhibitors, and agonists of the PAMP-associated pathway to characterize the mechanisms of OPC-mediated transcript stability. We identified a limited number of polyphenols that could stabilize the *GMCSF* 3'UTR, indicating this was a response distinct from the Nrf2 or 67LR pathway. Furthermore, we identified syk as a key signaling mediator of OPC-induced stability. Finally, we identified that the response to OPCs is dependent upon surface receptors, however priming agents including toll-like receptor agonists and similar PAMPs could not induce the transcript stabilization phenotype associated with OPCs. These results indicate OPC-mediated stability is a mechanism induced by select polyphenol agonists, and identified syk as a key component of this pathway. The activation of syk kinase by OPCs further develops the signaling pathway induced by these plant products.

## Methods

### Generation of the BE5B Reporter Cell Line

Whole virus for the *GFP-3'UTR-GMCSF* reporter was prepared using a third generation lentiviral expression system (Applied Biological Materials; Richmond, BC, Canada) and transfected into MOLT14 cells by spinocculation. Prior to transduction,

cells were rested overnight in complete RPMI [cRPMI; 10% serum (Fetal calf serum, Hyclone], RPMI 1040 with phenol red, HEPES, L-glutamine, non-essential amino acids, sodium pyruvate, penicillin and streptomycin, and MEM vitamins (all from Mediatech)) at $1x10^6$/mL. Lentiviral stock (1mL) was added to $1x10^6$ MOLT14 cells in 1mL cRPMI. Cells were centrifuged for 30m, medium replaced, and cultured for two weeks at 37°C and 10% $CO_2$. The single cell subclone BE5B was prepared by limiting dilution.

Preparation of Polyphenol
Extracts and Defined Chemicals

Polyphenol extracts for APP and GSE were purchased from Tianjin Jianfeng Natural Product R&D (Tianjin, China) and resuspended to 100mg/mL in water. Black Raspberry extract was kindly provided by Gary Stoner of Ohio State University (50mg/mL crude extract in DMSO) and Oenothein B was provided by Igor Schepetkin at Montana State University. Darjeeling tea was prepared by steeping 100g loose-leaf tea (Montana Tea and Spice Traders, Missoula MT) in 1L of 25°C water for 5m. The soluble fraction was separated by 2500 RPM centrifugation for 30m, and sequential filtering to 0.2µm using nylon syringe filters. Cocoa (*Theobroma cacao*) seed (Nestle Toll House, Vevey Switzerland) and Acai (*Euterpe Oleracea* BRS-Para) seed (AcaiPalmSeeds.com) polyphenols were prepared by methanol extraction. Acai seeds, with hair fibers removed, were crushed into small pieces until they were approximately 3 to 5mm in diameter and further ground in a food processor until they were a maximum of 1mm in diameter. 430g of germinated (less than 1 inch growth) and 140g non-germinated seeds were separated prior to extraction. The Acai seeds and cocoa seed powder were extracted with 2

volumes of MeOH treated overnight at 25°C. The soluble fractions were rezserved and the particulate re-extracted twice more. The MeOH solutions were centrifuged to remove particulate (7000RPM, Sorvall) and 0.2µm filtered before drying by vacuum concentrator (Savant SpeedVac; Thermo Scientific, Waltham, MA). Samples were then suspended in water to 100mg/mL and sonicated for 3m each, prior to 0.2µm filtration and drying. All extract stocks were re-suspended in water prior to immediate use or storage at -70°C.

Individual polyphenol species were prepared in solution to 50mg/mL or the highest concentration possible without heating: (-)Epigallocatechin (EGC; 10mg/mL in H2O. Chromadex, Irvine CA), Silychristin (800µg/mL in XVIVO medium. Chromadex), Theaflavin-3,3'-Digallate (TF3; 15mg/mL in EtOH. Chromadex), Resveratrol (50mg/mL in DMSO, Sigma St. Louis MO), Catechol (100mg/mL in H2O. Acros, Geel Belgium), (+)Catechin (Catechin; 10mg/mL in PBS. Cen-Med Enterprises New Brunswick, NJ), (-)Epicatechin, (Epicatechin; 10mg/mL in PBS. Sigma), Procyanidin B1 (PB1; 20mg/mL in water. Chromadex), Procyanidin B2 (PB2, 20mg/mL in water. Chromadex), Procyanidin C1 (PC1; 50mg/mL in water. PhytoLab; Vestenbergsgreuth, Germany or Cerilliant; Round Rock, TX), Rosmarinic acid (20mg/mL in DMSO, Sigma) and Gallocatechin Gallate (GCG, 1mg/mL in water, Sigma), and Epigallocatechin Gallate (EGCG; 1mg/mL in PBS or prepared fresh in culture medium. Sigma)

Kinase Inhibitor Impact on OPC-Induced Stability

To determine the pathways responsible for OPC-mediated transcript stability, small molecule inhibitors were used. Four treatment groups were prepared for each inhibitor,

APP/inhibitor, APP/vehicle, water/inhibitor, or water/vehicle. Inhibitor, or the appropriate vehicle, was prepared at 50-fold $IC_{50}$, and diluted 50% for a minimum of 8 total concentrations. The inhibitors used and their $IC_{50}$ values were: U0126 (MEK1/2, $IC_{50}$: 60nM Enzo Life Sciences, Farmingdale, NY or EMD, Darmstadt, Germany), syk inhibitor II, (syk, $IC_{50}$:41nM), H89(MSK1/PKA, $IC_{50}$:100nM, Abcam Biochemicals, Cambridge MA), CGP75380(MNK, $IC_{50}$: 2µM, Abcam Biochemicals), SB202190(p38, $IC_{50}$: 30nM), AS601245 (JNK, $IC_{50}$: 220nM), wedelolactone (IKK, $IC_{50}$: 10µM, in DMSO, CalBioChem), wortmannin (PI3K, $IC_{50}$ 50nM), BI-D1870 (RSK#1, $IC_{50}$: 31nM), SL0101 (RSK#2, $IC_{50}$:100nM, CalBioChem). Cells at a concentration of $2x10^6$ cells/mL were cultured in 96-well round bottom tissue culture plates (BD) with the inhibitors or their vehicle controls for 60m prior to the addition of water or 100µg/mL APP. After an additional 4h, cells were collected and analyzed by flow cytometry for GFP expression.

Screening of Biologically-Active Polyphenols

BE5B cells were cultured overnight in XVIVO (Lonza, Basel Switzerland). Cells were then added to agonists and cultured for an additional 4h before being centrifuged at 1500RPM for 5m to pellet cells. The cell pellets were then vortexed for 5s and re-suspended in cold PBS prior to reading or, in the case of the polyphenol screens, in cold PBS with 1% horse serum and 1% 7-AAD solution (eBioscience) to measure toxicity. After a 20m incubation at 4°C the cells from the polyphenol screens were pelleted by centrifugation, re-suspended in 200uL cold PBS with 1% horse serum, and again pelleted by centrifugation. The cells were then fixed with a cold 1% formaldehyde/2ug/mL

Actinomycin D solution in PBS. After 10m, cells were pelleted and re-suspended in 200uL cold PBS with 1% horse serum before reading on a flow cytometer (FACSCalibur with HTS option, Becton Dickinson). Live cells were selected based on FSC/SSC profile and 7-AAD- staining. All other cells were measured directly on the cytometer after washing with cold PBS/HS and live cells were gated by FSC/SSC profile). The median fold-increase in fluorescence intensity versus an in-plate vehicle only culture was used to determine changes in GFP expression. The coefficient of variance (CV) was determined for each series and the fold change.

## Results

### Treatment of MOLT14 with OPCs Increases Protein Expression by Prolonging Transcript Stability

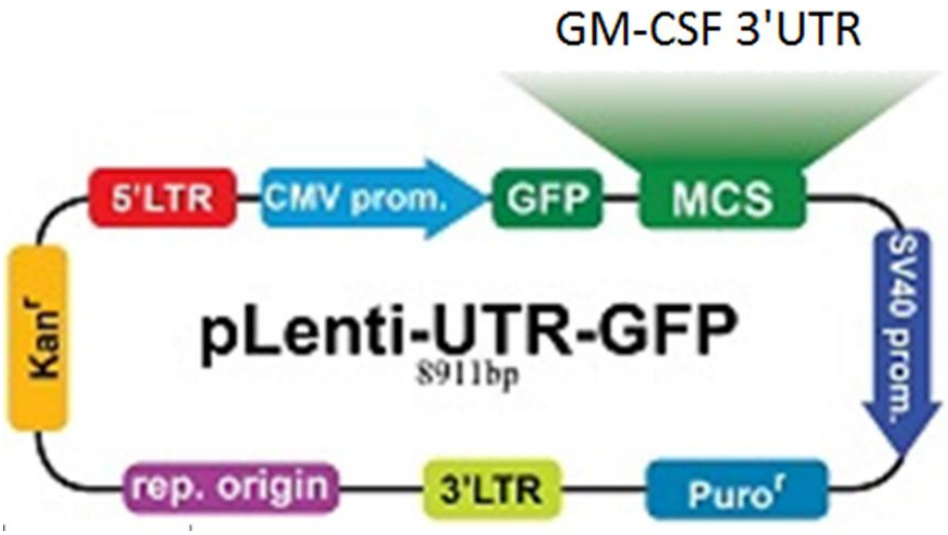
Previously we showed that OPCs from APP could increase the stability of select transcripts in primary γδ T cells from bovine or human origin. The stabilized transcripts included *GMCSF* and *IL8*, but not *JUN*, and this activity was dependent upon ERK kinase activity. To confirm that these transcripts were functionally active, we prepared polysome fractions of MOLT14 cells treated with APP, TNFα, or PBS for 4h before lysis and fractionation. qPCR analysis of *GMCSF* transcripts from sucrose density gradients identified transcripts in the polysome fractions from APP and TNFα-treated MOLT14 cells, indicating that *GMCSF* transcript was actively being translated (data not shown). Unfortunately, a complication with this cell line is that *GMCSF* transcript is not normally produced and must be induced before stability measurements can be made. Therefore,

we prepared a reporter construct based on constitutive GFP expression containing the 3'UTR of *GMCSF*. This line was developed to address two questions: First, does translational efficiency change during constitutive expression as opposed to induced? Secondly, is the transcript stability induced by OPCs dependent upon AU-rich elements in the 3'UTR of select genes as previously hypothesized?[250] The production of this reporter line allowed us to address both of these questions about OPC-mediated transcript stability.

For these studies, we desired a stable cell line so as to reduce variability from transient transfection and to provide a screening tool for down-stream applications. Therefore, we used a lentiviral vector to transfect the *GFP-3'UTR-GMCSF* into the MOLT14 cell line. This construct is under the control of the constitutive promoter CMV so that changes in protein level (i.e. fluorescence) would be indicative in changes to the degradation rate of the transcript (Figure 4.1). Analysis of the bulk transduction showed that GFP expression was highly variable; however, treatment with APP did increase the median fluorescence intensity, suggesting that APP-mediated stabilization resulted in increased GFP protein (data not shown). To obtain a reporter line expressing more consistent levels of GFP at baseline, a single cell clone, BE5B, was isolated.

We next used the BE5B reporter line to measure time and dose-dependent responses to APP. First, cells were cultured with 0 to 300μg/mL APP for 4h, collected, and then analyzed for GFP expression by flow cytometry (Figure 4.2A). These experiments demonstrated a dose-dependent increase in GFP expression in BE5B cells when treated with APP, suggesting the transcript stabilizing effects of OPCs leads to an increase in

Figure 4.1 Lentiviral Vector Map for the *GFP-GMCSF3'UTR* Reporter




available mRNA for translation. Next, we sought to characterize the amount of time required for OPC-mediated transcript stabilization to elicit changes in protein expression. To this end, we treated BE5B cells with 100µg/mL of APP for 0m to 240m. 100µg/mL was selected for these studies because it was able to induce a robust increase in GFP expression in Figure 4.2A and because our previous studies in MOLT14 cells showed very little toxicity at this concentration over culture periods of up to 24h in XVIVO (unpublished data). Similar to our previous observation that OPCs can induce rapid changes to *GMCSF* transcription, translation efficiency increased in as little as 60m (Figure 4.2B). Importantly, these experiments also showed that the addition of APP did not significantly alter fluorescence alone (0m APP versus medium-only control). These results showed that OPC-mediated transcriptional stability results in increased

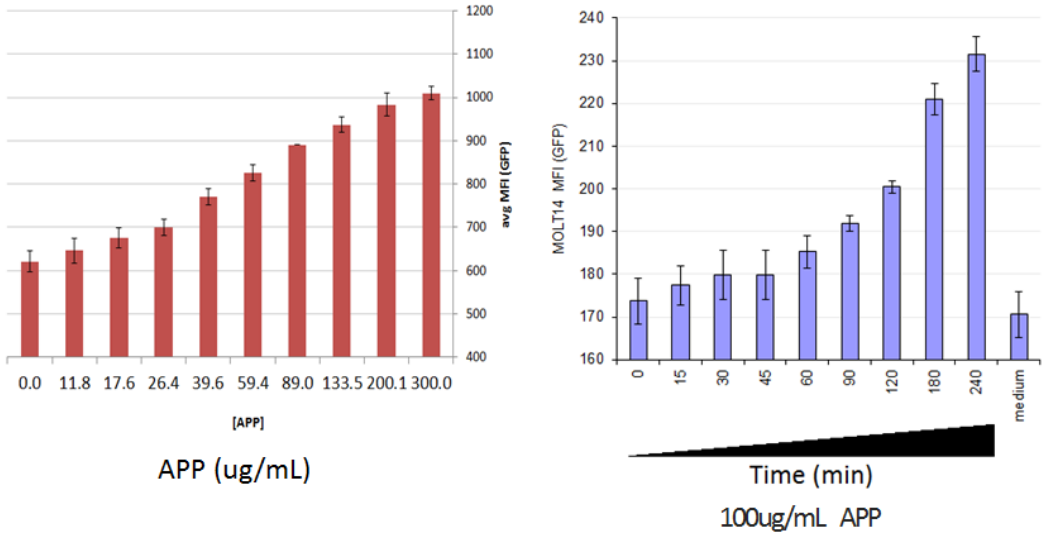
translational efficiency, that is dependent upon the 3'UTR of select transcripts, and that BE5B functions as a reporter assay to measure the effects of OPCs on transcript stability.

## Transcript Stabilization is a Response Mediated by Select Polyphenols.

The efficiency of the BE5B cell line in measuring OPC-mediated transcript stabilization prompted us to determine if this phenomenon was restricted to OPCs or whether other polyphenols with described immunostimulatory activity could generate comparable responses. Therefore, plant extracts or polyphenol preparations with known immunomodulatory activity were tested for transcript stabilizing activity. In these studies, we included extracts from black and green teas, numerous OPC sources, and a black raspberry extract. For the black tea samples, we tested an aqueous extract as well as theaflavin 3,3'-digallate (TF3). For the green tea polyphenols, we tested catechin, epicatechin, EGCG, GCG, and EGC. A black raspberry extract which is composed predominantly of anthocyanin species,[328] was also tested. Finally, we tested OPC-rich samples for activity. These included APP, grape seed extract (GSE), and seeds from the Acai fruit. In addition, we included monomers from the OPCs, epicatechin and catechin, the dimers PB1 and PB2, and the trimeric OPC, PC1. We also included Oenothein B, a polyphenol with innate stimulatory activity.[329] Included in these samples were agonists reported to activate the 67LR ligand (EGCG)[19] and the Nrf2 pathway, (resveratrol).[20] We identified OPCs and Oenothein B as candidates for stabilizing transcript stability, but other agonists did not demonstrate comparable responses. These results indicate that

OPC-mediated transcript stability is a phenomenon induced by limited number of polyphenol agonists and that the Nrf2 and 67LR agonists do not induce this response.

Figure 4.2 The MOLT14 *GFP-3'GMCSF UTR* Reporter Line Efficiently Represents Procyanidin-Induced Transcript Stability




The BE5B clone from the transduced MOLT14 cell line expressing GFP-3'UTR-GMCSF was tested for its ability to increase GFP expression as a result of transcript stabilization during OPC culture. Cells were treated with various concentrations of the OPC, APP, and at various time points prior to analysis by flow cytometry. A) Changes in GFP expression were measured after 4h culture with varying doses of APP. B) GFP expression in BE5B cells was measured over time after 100µg/mL APP culture. Results represent the median fluorescence intensity (MFI) and error bars represent standard deviations of the triplicate cultures assayed for each treatment.

ERK and Syk Function in OPC Responses

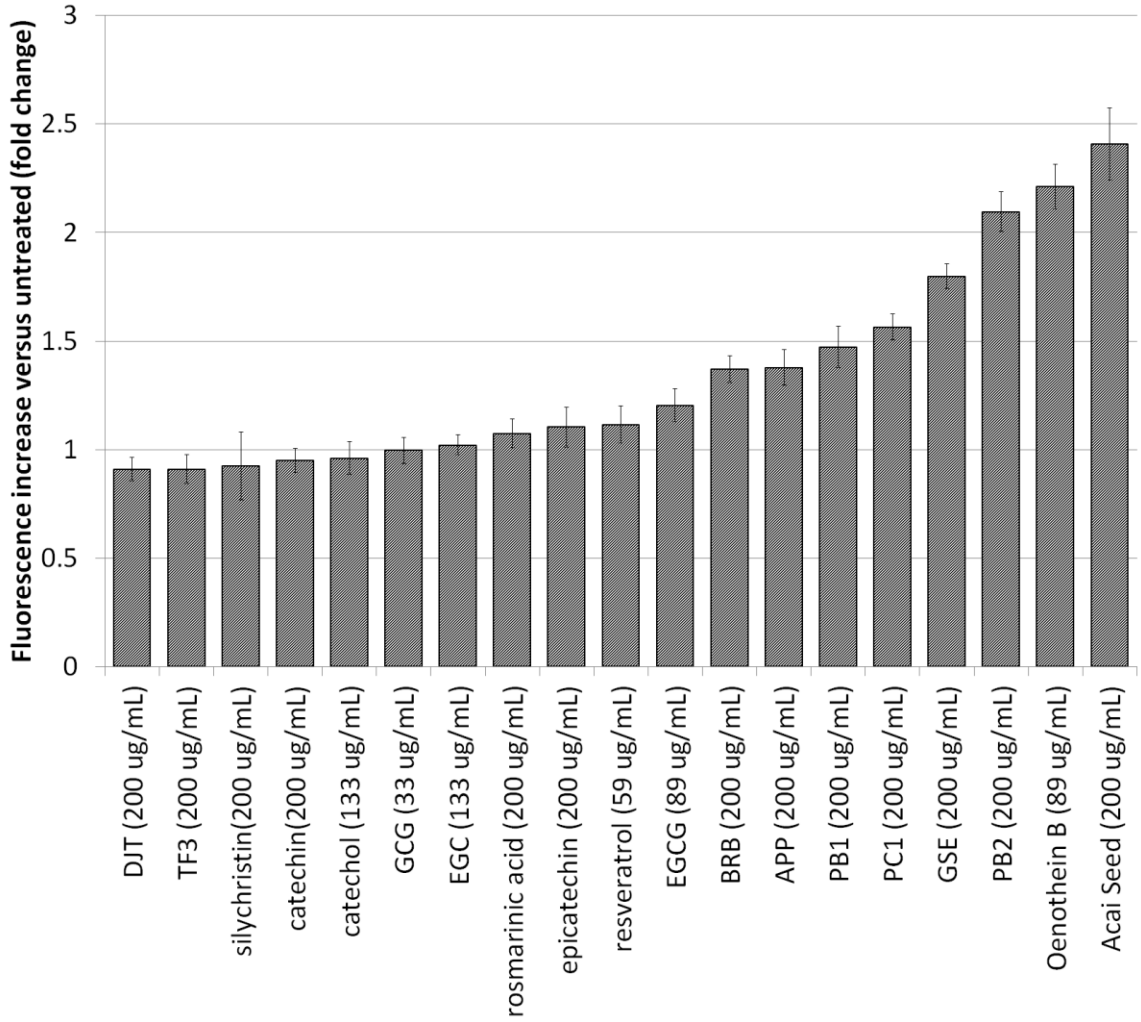
These results indicated that OPCs activated transcript stabilization through a mechanism independent of the described receptor-mediated pathways, LR67 and Nrf2. Therefore, we sought to further characterize the signaling pathway(s) utilized by OPCs for their function. We tested inhibitors for the kinases ERK, p38, c-Jun, IKK, RSK,

P13K, and syk as well as inhibitors for the ERK-downstream kinases PKA/MSK1, MNK, and RSK. As previously shown, ERK inhibited OPC-mediated transcript stability (Figure 4.4A), but of the other inhibitors, only inhibition of syk reproducibly attenuated OPC-induced stability (Figure 4.4B). These experiments indicate that, in addition to ERK, the transcript stabilizing effects of OPC are mediated by the kinase syk.

## Discussion

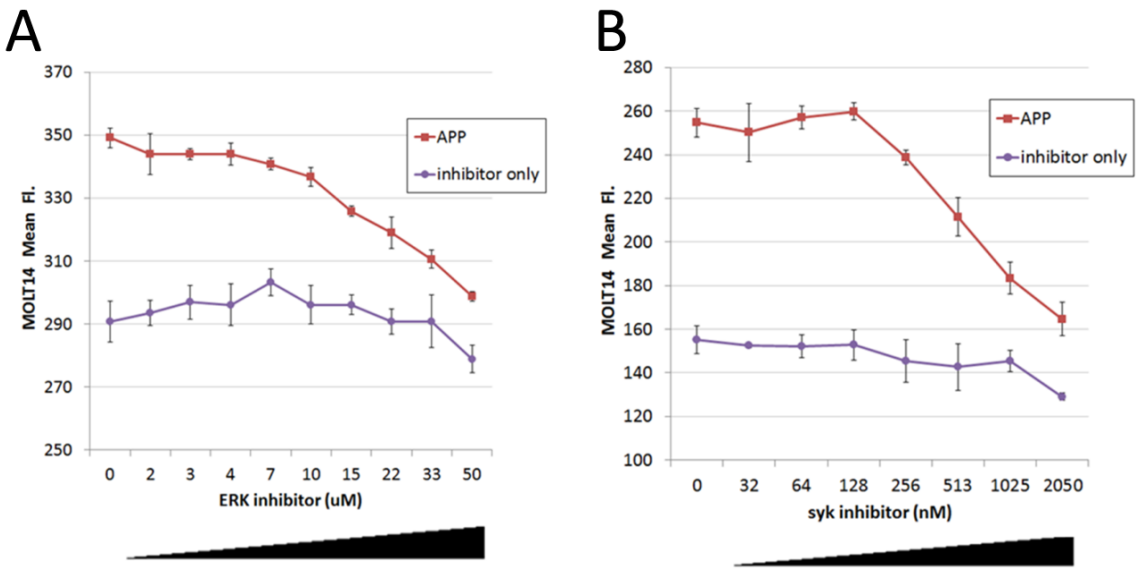
Multiple plant polyphenols are ascribed with immunomodulatory activity; however, the cellular mechanisms induced by these molecules are rarely described. Previously, we identified the ability of OPCs from APP and a trimeric OPC, PC1, to stabilize select transcripts in the γδ T cell line, MOLT-14.[250] As a continuation of these studies, we developed the BE5B reporter line to measure activity in other polyphenol preparations. We found that the ability to stabilize transcripts containing the 3' UTR from *GMCSF* is largely restricted to the OPCs. Interestingly, Oenothein B, another γδ T cell agonist, was able to increase GFP expression in the MOLT-14 reporter line. Oenothein B's structure is very different from OPCs, it is a cyclic hydrolysable tannin dimer.[330] Whether this polyphenol activates similarly to OPCs was not studied here, but the increased GFP expression during culture remains an interesting observation. Future studies will look at whether this apparent mimic of OPC activity likewise leads to increased transcript stability and whether it uses the same pathways to mediate this response.

Figure 4.3 Stabilization of *GMCSF* 3'UTR by Polyphenols




BE5B cells were cultured for 4h with various plant extracts or polyphenols. Cultures at the highest concentration tested, up to 200µg/mL, that did not induce more than 20% cell death were measured for GFP expression by flow cytometry. Fold increase was calculated from an in-plate vehicle-only control. Error bars represent the CV from triplicate values of each vehicle and agonist concentration. Results are representative of two experiments.

Figure 4.4 ERK or Syk Inhibition Attenuates OPC-Induced Transcript Stability




BE5B cells were pretreated for 1h with various concentrations of inhibitors for ERK or syk, and then treated for 4h with either 100ug/mL APP or water as a vehicle control. A) ERK kinase inhibitor, U0126 (inhibits the upstream kinase MEK1/2. The ideal $IC_{50}$ for this inhibitor is 72nM. The optimal reported concentration in cell culture for inhibition is 50µM)[331] B) syk inhibitor (Syk inhibitor II, $IC_{50}$ 41nM for syk, PKCε; 5.1µM, others >11µM). The optimal reported concentration for use in cell culture is 1µM).[332]

The generation of the BE5B cell line has many advantages over our previous methods of visualizing OPC-induced transcript stability. It allows us to measure the presence of transcripts without the potential complications of chemically-inhibited transcription, the need to prepare RNA samples, and the expense of qPCR. The BE5B cell line is also a more rapid screening tool for analyzing the effects of OPCs. As is shown in Figure 4.2B, changes in GFP expression can be detected in as little as 60m, and this can be rapidly analyzed by flow cytometry. Perhaps the largest advantage of this cell line over the chemically-inhibited method is the ability to detect changes to stability at low

concentrations. OPCs can be toxic to cell lines near the concentrations required to induce GM-CSF.[250] Figure 4.2A shows that we detect transcript stabilization in the BE5B cell line at a wide range of OPC concentrations, including those that are not able to reliably induce *GMCSF* transcript. These advantages provided by this reporter cell line have enabled us to use it as a quantitative assay for the rapid screening of potential agonists and the pathways involved in these responses.

The absence of activity in the other polyphenol agonists suggests that OPCs do not activate either the Nrf2 or the LR67 pathway. Or, if they do, this activity is separate from transcript stabilization. Others have shown that OPCs from grapes do not activate the Nrf2 pathway.[333] Likewise, we did not see an increase in the Nrf2-associated genes, *CYP1A1* or *CYP1B1* (unpublished data), or in the Nrf2-associated MAPK, p38.[250] On the other hand, some groups show that dimeric and oligomeric OPCs, are able to activate Nrf2-associated transcripts in some settings. These studies rely on Hep2a or CACO-2 reporter lines, which are very different than the cell types we work with. As is expected for Nrf2 activation, these responses were dependent upon p38 for both of these studies, however, ERK was dispensable in one and not the other.[334,335] Thus, the potential of OPCs to activate Nrf2 is still unclear, and may be dependent upon the cell lines or OPC concentrations used. In spite of the uncertainty regarding Nrf2, the experiments within this study suggest that OPCs, but not Nrf2 agonists, induce the stabilization of transcripts, and thus stabilization is a response independent of the Nrf2 pathway.

We used pathway-specific inhibitors to determine the signaling pathways involved in OPC-mediated transcript stability. Our initial report identified the MAPK ERK as

critical to stabilization of the *GMCSF* transcript. The BE5B cell line reproduced our previous transcript stabilization results, except by using GFP expression this allowed us to more accurately measure temporal changes to transcript stability and test multiple kinase pathways for their involvement. In these studies, we identified the kinase, syk, as a critical mediator of OPC-induced transcript stabilization. Syk is commonly associated with phagocytic responses from lectin receptors, but is also involved in CD14-mediated endocytosis of LPS,[61] Fc Receptor activation, B-cell receptor (BCR), and G-coupled receptors.[336] How these play a role in the response to OPCs remains to be determined, yet they further narrow the field of potential surface receptors that may be triggered by OPCs.

The efficiency of using this cell line as a tool for measuring OPC-mediated transcript stability has enabled us to rapidly screen additional polyphenols for activity and to characterize some of the molecular mechanisms mediating this response. These results show that this response is attributed to only the OPCs and Oenothein B, but not the more commonly described plant polyphenols. The identification of this unique response has encouraged us to develop this assay as a platform amenable to quality control testing in the neutraceutical industry. In combination with other screening assays in development for measuring responses to other polyphenols,[337] these assays could provide a thorough analysis of the immunologic attributes for a given neutraceutical preparation. This would far out-value the current use of Oxygen Radical Absorbance Capacity (ORAC) as a measure of neutraceutical activity, which is, in fact, emphasized as inappropriate by clinical studies and the European Food Safety Authority.[214,215,338]

# CHAPTER FIVE

## POLYSACCHARIDES ISOLATED FROM ACAI FRUIT INDUCE INNATE IMMUNE RESPONSES

### Contribution of Author and Co-Authors

Manuscript in Chapter Five

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Contributions: Designed and performed experiments, prepared the manuscript.

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Contributions: Designed and performed experiments, prepared the manuscript.

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Contributions: Designed and performed experiments.

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Contributions: Designed and performed experiments.

Co-author: Mark T. Quinn

Contributions: Obtained funding and edited the manuscript

Co-author: Jodi F. Hedges

Contributions: Designed experiments and edited the manuscript

Co-author: Mark A. Jutila

Contributions: Designed experiments, obtained funding, and edited the manuscript.

Manuscript Information Page

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## Abstract

The Açaí (Acai) fruit is a popular nutritional supplement that purportedly enhances immune system function. These anecdotal claims are supported by limited studies describing immune responses to the Acai polyphenol fraction. Previously, we characterized γδ T cell responses to both polyphenol and polysaccharide fractions from several plant-derived nutritional supplements. Similar polyphenol and polysaccharide fractions are found in Acai fruit, as such, we hypothesized that one or both of these fractions could activate γδ T cells. Contrary to previous reports, we did not identify agonist activity in the polyphenol fraction; however the Acai polysaccharide fraction induced robust γδ T cell stimulatory activity in human, mouse, and bovine cultures. To characterize the immune response to Açaí (Acai) polysaccharides, we fractionated the crude polysaccharide preparation and tested these fractions for activity in human PBMC cultures. The largest Acai polysaccharides were the most active *in vitro* as indicated by activation of myeloid and γδ T cells. When delivered *in vivo*, Acai polysaccharide induced myeloid cell recruitment and IL-12 production. These results define innate immune responses induced by the polysaccharide component of Acai and have implications for the treatment of asthma and infectious disease.


## Introduction

Herbal products have been used in traditional medicines for a variety of infectious and inflammatory diseases. Many of these plant materials enhance the activity of cells of

the innate immune system and modify host responses.[1,2,4,339,340] Though their use is widespread, in many instances the responding aspects of the immune system are unknown and the optimal therapeutic potential of these materials unrealized. Most studies describing the innate immune response to herbal products focus on cells of the myeloid lineage, such as macrophages and neutrophils. Our studies incorporate an additional cell type, the γδ T cell, which responds to at least three distinct herbal components.[1,2,341] γδ T cells are well placed to respond to traditional medicines as they are found in the gut mucosa and virtually all portals of entry in the body. These T cells contribute to effective innate immune responses against a variety of infectious agents [206,342-345]. They also facilitate downstream adaptive immune responses, due in part, to cytokine production[346-348] and stimulation of dendritic cell function.[349,350] γδ T cells are also potent cytolytic cells,[351,352] can present antigen,[353,354] induce or suppress inflammation,[355-357] and are important to the health of epithelial cell monolayers.[134,358] Due to the abundance of functional responses elicited by this cell type, its role in response to traditional medicine may have clinical relevance. In fact, novel therapeutic protocols, developed from natural agonists for the γδ T cell, are being pursued in the clinic to combat infection and cancers.[359,360]

The consumption of many traditional medicines is associated with the induction of γδ T cell activity. For example, the health benefits of tea consumption can be linked to stimulation of the human γδ T cell anti-microbial response.[361,362] Additionally, some fruit and vegetable juices expand γδ T cells following consumption.[363,364] Recently, we

elaborated upon this association between traditional medicines and γδ T cell activity by defining immunomodulatory components within common traditional medicines.

Thus far, three functionally and structurally distinct plant-derived agonists capable of inducing γδ T cell responses are described: prenyl phosphates, polyphenols, and polysaccharides. Prenyl phosphates derived from plant and microbial sources rapidly expand a subset of human γδ T cells, but function only in primate species.[126,341] The polyphenol agonists act directly on the γδ T cell by inducing a priming response, typified by up-regulation of activation markers and an increased responsiveness to secondary stimuli.[1] While γδ T cell activating polyphenols are found in several plants,[2] the polyphenol agonists are best illustrated with non-ripe apple peel polyphenols (APP).[1] The most active polyphenol fraction identified consists of oligomeric procyanidins (OPCs),[2] however, other polyphenols including Oenothein B contain agonist activity,[329] suggesting polyphenol agonists are structurally heterogeneous. The final type of plant product with γδ T cell agonist activity includes polysaccharides from Yamoa™, the ground bark of the *Funtumia elastica* tree.[1,4] Yamoa polysaccharides (referred to herein as Yam-1) induce robust *in vitro* effects on γδ T cells from bovine calves, humans, and mice. While γδ T cells respond directly to Yamoa polysaccharides, these responses are greatly amplified during monocyte/macrophage co-culture.[4] As a caveat to our understanding its activity, Yamoa exhibits reactivity in the limulus amebocyte lysate (LAL) assay. For this reason, separating polysaccharide agonist activity from the endotoxin activity in this preparation is problematic. Nonetheless, there is an endotoxin-

independent component of Yamoa as evidenced by a retained response in MyD88$^{-/-}$ and TLR4$^{-/-}$ mice.[4]

The fruit from Acai, *Euterpe oleracea*, has become a popular nutritional supplement with anecdotal claims in support of immune stimulation. Since Acai contains both polyphenols and polysaccharides, it was tested for γδ T cell agonist activity. Whereas others have reported that the major polyphenol components from Acai contain immunomodulatory functions,[365-369] we found that the polysaccharides, and not the polyphenols, from Acai stimulated bovine, mouse and human γδ T cells in mixed leukocyte cultures. Acai-derived polysaccharides also stimulated monocytes/macrophages, which was not due to endotoxin contamination since the Acai fractions were found to contain very low LAL activity and were similarly active after polymyxin B treatment to remove residual endotoxin. Moreover, we evaluated pro-inflammatory responses to Acai polysaccharides *in vivo;* after either intraperitoneal or intratracheal administration of Acai polysaccharides, neutrophil recruitment to the respective tissue was observed. Furthermore, delivery of Acai polysaccharide to the lung activated alveolar macrophages and induced IL-12 production. Overall, these results indicate that the polysaccharide fraction is responsible, at least in part, for the immune responses reported for Acai and underscore its potential use as a therapeutic or prophylactic treatment for infectious disease.

## Materials and Methods

### Ethics Statement

All experiments were performed in accordance with National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee (protocol identification: 2008-15, 2009-3). Human subjects testing was performed in accord with the Institutional Review Board of Montana State University (approval identification: MJ032609) and written, informed consent was obtained from all individuals.

### Polysaccharide Isolation from Acai

Acai fruit pulp was obtained from two sources: Acai 100 (Genesis Today; Austin, TX) and Acai Berry Pure (Acai Berry Pure Bulk; Carlsbad, CA). The Acai 100 preparation consisted of 100% pure, liquid-format Acai fruit and was used to obtain preliminary results (data not shown) as well as to prepare the preliminary EtOH precipitation and Kupchan fractionation[370] products (prepared by contract: PhytoMyco Research Corporation; Greenville, North Carolina).

All other experiments utilized Acai Berry Pure. Polysaccharides were isolated from this powdered Acai as described previously.[4] Briefly, 1500g of Acai powder was extracted with 8L boiling, distilled $H_2O$ for 1h. The aqueous extract was then centrifuged at 2,000 × g for 15m, and a 4-fold volume of ethanol was added to the supernatant to precipitate polysaccharides overnight at $4^oC$. The precipitate was pelleted by centrifugation, re-dissolved in distilled $H_2O$, and centrifuged at 2,000 × g for 15m. The supernatant fluid (crude polysaccharide extract) was fractionated using ion-exchange

chromatography on a DEAE-cellulose column equilibrated with 0.05M Tris-HCl buffer (pH 8.0). Bound material was sequentially eluted with 0.05M Tris-HCl buffer and 2M NaCl; a recovery of 0.27% by weight or 4g total weight was achieved. The presence of polysaccharides in the unbound fraction, eluted with 0.05M Tris-HCl buffer was minimal (<0.1% of total bound fraction). The AcaiPS fraction was generated from the bound material after concentration in an Amicon concentrator with a 10kDa Amicon PM10 membrane (Millipore; Billerica, MA). Further fractions were produced by size exclusion chromatography on a Sepharose-6B column (2.5×95 cm) equilibrated with 0.01M Tris-HCl buffer (pH 7.2) containing 0.15M NaCl and eluted with the same buffer at a flow rate of 22mL/h. The relevant fractions were pooled and concentrated. Three fractions were obtained, designated as Acai-1 (0.7g total weight), Acai-2 (1.5g total weight), and Acai-3 (0.85g total weight). These fractions were analyzed by HPLC, and elution was monitored with a refractive index detector as described previously [7].

Polyphenol Isolation and Removal

Acai polyphenols were extracted from dried fruit pulp using the method described by Rodrigues *et al* [369]. Briefly, 100g of Acai fruit (Acai Berry Pure) was washed over a three day period with exchanges of 350mL, 350mL, and 300mL of MeOH. Next, the MeOH-extracted material was dried using a Savant SpeedVac® Plus SC210A Concentrator (Thermo Scientific; Waltham, MA). To isolate polyphenols, 5g of polyvinylpolypyrrolidone (PVPP; Sigma-Aldrich, St. Louis, MO), triple-washed in water, was added to 5mL of 24mg/mL water-reconstituted, MeOH-extracted Acai. Polyphenols

were allowed to adsorb to the PVPP matrix for 10min prior to triple-washing with 20mL DPBS. Polyphenols were eluted with 10mL of 0.5N NaOH for 5min. The resulting polyphenols were adjusted to approximately pH 7.0 with HCl, dried to determine weight, and tested for their ability to stimulate human peripheral blood mononuclear cells (PBMCs).

For a second approach to measure polyphenol contribution, AcaiPS (100mg) was transferred over a column containing 2g of triple-washed PVPP. The eluent was filtered through a 0.2µm filter and similarly assessed for stimulatory activity.

Characterization of AcaiPS Fractions

The presence of type II arabinogalactan structures was detected by single radial diffusion in a 1% agarose gel containing 0.1mg/mL β-glucosyl-Yariv reagent, 4-β-d-glucopyranosyl oxyphenylazo-2,4,6-trihydroxybenzene (Biosupplies; Melbourne, Australia), which specifically interacts with and precipitates compounds containing type II arabinogalactan structures. A solution of 2mg/mL arabic gum (Fluka; St. Louis, MO) in $H_2O$ was used as a standard, and the polysaccharide samples were tested at 2mg/mL. After application of 6µL samples, the gels were incubated for 24h at room temperature in a humid atmosphere. Arabinogalactan-positive reactions were identified by a reddish circle (halo) around the wells.

Fluorescence measurements were performed using an LS50 luminescence spectrometer (Perkin Elmer). Samples were dissolved in $NaHCO_3$ (25mM, pH8.5).

Synchronous fluorescence spectra were recorded from 300 to 600nm at a scan rate of 240nm/min. The excitation–emission wavelength difference ($\Delta\lambda$) was 20nm.

Protein content was measured using the Bradford method as per the manufacturer's directions (Bio-Rad Protein Assay: Bio-Rad; Hercules, CA). Bovine serum albumin was used to generate a standard curve. Absorbance was measured at 595nm using a SpectraMax Plus microplate reader (Molecular Devices; Sunnyvale, CA).

The approximate molecular weight of the AcaiPS fractions was determined by high performance size exclusion chromatography (HP-SEC) using a Shimadzu Class VP HPLC and Shodex OHpak SB-804 HQ column (8mm x 300mm) as previously described.[8] The molecular weights were estimated by comparison to the retention times of pullulan polymer standards (P-800, -400, -200, -100, -50, -20, and -10; Phenomenex, Torrance CA).

Monosaccharide analysis was performed by the Oklahoma Center for Glycobiology Analytical Core Lab (Oklahoma City, OK). Briefly, polysaccharide samples or background blanks were subjected to methanolysis (methanolic 2M HCl, 16h, 80°C), followed by acid hydrolysis (2M trifluoroacetic acid, 2h, 100°C), and the resulting monosaccharide mixtures were analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a Dionex DX-600 HPAEC system equipped with an ED50 detector (Dionex Corporation; Sunnyvale, CA). The samples were separated on a Dionex CarboPac PA-1 column eluted isocratically with 6mM or 26mM NaOH for 30min, then a 100mM NaOH gradient for 10min followed by a sodium acetate gradient from 0 to 500mM for 35min at a flow rate

of 1mL/min at 22°C. For analysis of uronic acids, the column was eluted with 10mM NaOH for 20min, followed by a gradient of 100mM NaOH/150mM sodium acetate (0–100% for the duration of 45min). Background signals were subtracted from all samples, and individual components were quantified based on electrochemical detection relative to known standards.[371,372]

Carbohydrate content was determined for AcaiPS by phenol-sulfuric acid method, modified to a microplate format,[373] and absorbance was measured at 490nm using a SpectraMax Plus microplate reader. A solution was prepared based on Acai-1 sugar content as: 4.5% L-Rhamnose (Sigma-Aldrich), 47.0% L-(+)-Arabinose (Sigma-Aldrich), 11.5% D-(+)-Galactose (Sigma-Aldrich), 2.8% D-(+)-Xylose (Sigma-Aldrich), 28.4% D-(+)-galacturonic acid (Fluka), and 3.0% D-Glucuronic acid (Sigma-Aldrich) by weight solution in DPBS. This solution was used to generate a standard curve.

The total amount of polyphenols in the Acai fractions was determined by Folin-Ciocalteu assay[374] as previously described [1]. Briefly, 250µL of Folin's phenol reagent was added to the samples dissolved in 500µL distilled water. After 3min at room temperature, 1.25mL of 20% sodium carbonate was added, mixed, and the mixture was allowed to stand for 40min. The absorbance was measured at 750nm in a spectrophotometer (DU800: Beckman Coulter; Brea, CA). The total polyphenol content was determined using epicatechin to generate a standard curve and expressed as epicatechin equivalents (epicatechin/mg sample) × 100.

LAL assay was used to estimate the amount of endotoxin contained in the polysaccharide fractions from Acai. For all samples tested except the crude Acai EtOH

precipitation, a Pyrochrome LAL reagent reconstituted with Glucashield in an endotoxin-free microplate (all from Associates of Cape Cod; East Falmouth, MA) as per manufacturer's procedures was used. Analyses of endotoxin concentration were performed via the kinetic method using a VersaMax plate reader with SoftMax Pro software (Molecular Devices). The crude EtOH-precipitated Acai was tested for LAL reactivity using the PYROGENT (Cambrex; Charles City, IA) 0.125 EU/mL sensitivity inverted tube assay. To calculate the potential endotoxin content in Acai-1, Acai-2, and Acai-3 a ratio of 8EU/ng was used.

Endotoxin Removal

To remove potential contaminating endotoxin Acai-1 was applied to a column containing Detoxi-Gel Endotoxin Removing Gel (Pierce; St. Louis, MO) and eluted with 0.05M phosphate buffer containing 0.5M NaCl to decrease ionic interactions of sample molecules with the affinity ligand. The concentration of polysaccharides in the eluted sample (Acai-$1^{ER}$) was adjusted to match that of the untreated fraction (Acai-1), as determined by diene group content (absorbance at 254nm).[375]

Cell Cultures

All cells were cultured at 37$^{o}$C in a humidified atmosphere containing 5% $CO_2$. Human monocyte-macrophage MonoMac-6 cells (DSMZ; Brunswick, Germany) were grown in RPMI 1640 (Mediatech Inc.; Herndon, VA) supplemented with 10% (v/v) FBS, 10μg/mL bovine insulin, 100μg/mL streptomycin, and 100U/mL penicillin.

For primary cells, whole blood was collected from 1-3 month old bull Holstein calves into sodium heparin tubes (Becton Dickinson; Franklin Lakes, NJ) or healthy human adult donors with ACT tubes (Becton Dickinson). Peripheral blood mononuclear cells (PBMCs) were separated from whole blood using Histopaque 1077 (Sigma-Aldrich) for bovine cells as previously described [113] and human cells, as per the manufacturer's instructions. Additionally, bovine red blood cells were removed by hypotonic lysis. Preparation of spleens from TCR$\alpha^{-/-}$ mice for *in vitro* culture was performed as previously described.[4] Briefly, spleens were aseptically removed from the mouse, dounce homogenized, cold ACK buffer (8.29g/L $NH_4Cl$, 1g/L $KHCO_3$, 292mg/L EDTA)-treated for 10min, Nitex® filtered, and density separated using Lympholyte M (Cedarlane Laboratories; Burlington, NC) prior to culture at 2.5E$^6$/mL in XVIVO-15 medium (Lonza; Walkersville, MD). Splenocytes were infused with CFSE, cultured for 24h with agonists, washed with fresh medium, and re-cultured for 72h with medium containing recombinant murine IL-2 (rmIL-2; PeproTech; Rocky Hill, NJ).

Measurement of Cell Activation by Flow Cytometry

Flow cytometry was used to analyze cell activation in bovine, human, and mouse cultures as previously described.[1,2] To measure activation, cells were stained with anti-γδTCR monoclonal Ab (GD3.8[bovine],[376] GL3 [mouse; Becton Dickinson],[377] or 5A6.E9 [human; ATCC])[378] and either CD69 (human, FN50; Biolegend, San Diego, CA) or IL-2Rα/CD25(bovine, LCTB2A, VMRD, Pullman, WA).[379] For human activation assays, anti-CD19 (HIB19, Biolegend) and -CD3(UCHT1, Biolegend) were also used to

identify lymphocyte populations as follows: αβ T cells ($CD3^+$, $\gamma\delta TCR^-$), B cells ($CD3^-$, $CD19^+$), γδ T cells ($CD3^+$, $\gamma\delta TCR^+$), other cells ($CD3^-$, $CD19^-$, $\gamma\delta TCR^-$).

To measure mouse splenocyte proliferation in response to rmIL-2, they were stained with CFSE prior to culture as described[4] and stained with anti-γδ TCR mAb after culture. To differentiate mAb staining, FITC, PE, PE-Cy5.5, or Allophycocyanin (APC) fluorochromes were directly conjugated except for mAbs 5A6.E9 and LCTB2A, which were detected using fluorochrome-labeled goat-anti-mouse polyclonal Ab (Southern Biotech; Birmingham, AL). Indirect Ab staining was blocked using mouse serum before the addition of other Abs. Cells were analyzed using a FACSCalibur system equipped with a high-throughput sampler (Becton Dickinson).

### Analysis of Reactive Oxygen Species (ROS) Production

ROS production was analyzed using the chemiluminescent probe, L-012, which is highly sensitive for ROS generated in biologically complex systems.[380,381] Human PBMCs ($2\times10^5$ cells in 100µL per well) were incubated with various concentrations of polysaccharide fractions or positive control LPS for 24h. After incubation, culture supernatant fluid was replaced with an equal volume of HBSS supplemented with 25µM L-012 as described previously. The reaction was monitored on a Fluoroscan Ascent FL microtiter plate reader (ThermoElectron; Milford, MA) at 37°C. Chemiluminescence was measured every 2min for 3h and is expressed as the integrated response over this time (arbitrary units).

Determination of Acai-induced Cytokine Production

Cells were incubated for 24h in culture medium supplemented with 3% (v/v) endotoxin-free FBS, with or without Acai polysaccharide fractions or LPS as a positive control. Human PBMCs and MonoMac-6 human monocytic cells were plated in 96-well plates at a density $2\times10^5$ cells in 100µL per well. A human cytokine Multi-Analyte ELISArray™ Kit (SABiosciences Corporation; Frederick, MD) was utilized to evaluate various cytokines [interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, interferon-γ (IFNγ), tumor necrosis factor α (TNFα), and granulocyte-macrophage colony-stimulating factor (GM-CSF)] in supernatants of PBMCs. These results were confirmed using cells from a different donor with enzyme-linked immunosorbent assay (ELISA) kits for GM-CSF, TNFα, IL-1β, IL-6, and IL-8 (all from Biolegend).

Human TNFα or IL-6 enzyme-linked ELISA kits (Becton Dickinson and Biolegend) were used to quantify TNFα or IL-6 levels in the cell supernatants of PBMC or MonoMac-6 cultures.

Peritonitis Assay

BALB/c mice were injected intraperitoneally (i.p.) with 5µg Yam1,[4] 5µg or 50µg of Acai-PS, or saline only. After 4h, the mice were euthanized and the peritoneal cavity was washed with 10mL HBSS (injected and retrieved) and cells collected. The number of neutrophils was quantified by mAb stain: $CD45.2^+$ (104, Becton Dickinson), $CD11b^+$(M1/70, Becton Dickinson), and granulocyte receptor-1 (Gr-1;$RB6\text{-}8C5^{bright}$).[382] A known concentration of APC-labeled FACSbeads (Becton Dickinson) was added to

these cells prior to flow cytometry using a FACSCalibur equipped with a high-throughput sampler (Becton Dickinson). Viable leukocytes were gated based on FSC/SSC and positive CD45 staining. The absolute count of neutrophils was calculated based on the number of beads collected versus the number of viable neutrophils and extrapolated for the 10mL wash volume.

Lung Inflammation Assay

BALB/c mice (9-10 weeks, female) were instilled intratracheally (i.t.) with 1.56-500µg Acai-PS 24h prior to collection of bronchoalveolar lavage fluid (BALF) and lung tissue. Prior to tissue collection, mice were euthanized by $CO_2$ asphyxiation. BALF was collected by lavage with two 1mL washes of Hanks containing 2mM EDTA. Approximately 1.5mL lavage fluid was recovered from each wash. BALF was centrifuged and the supernatant fluid was saved for IL-12 ELISA (IL-12(total); C15.6/C17.8-biotin, MabTech; Nacka Strand, Sweden). The pelleted BALF cells were treated for 10min with cold ACK buffer to lyse red blood cells prior to analysis by flow cytometry. Next, lung tissue was collected by mincing with scissors then digestion for 1h in collagenase/DNase medium [200U/mL collagenase (Worthington Biochemical; Lakewood, New Jersey) and 0.08U/mL DNase (Promega; Madison, Wisconsin) in RPMI with 20mM HEPES] at 37$^{o}$C. The resulting product was then passed through 35µM Nitex® nylon mesh (Sefar America; Depew, NY) to remove tissue debris and ACK lysed. To analyze the cellular composition of the tissue and BALF, cells were stained

with CD11b-FITC (M1/70; Becton Dickinson) and CD11c-APC (HL3; Becton Dickinson) prior to analysis using a FACSCalibur cytometer (Becton Dickinson).

Statistics

Statistical analyses were performed using Microsoft Excel or GraphPad Prism.

## Results

### Acai Polysaccharides Induce Minimal Responses in LAL Assays and are Potent Agonists for Bovine, Human and Mouse γδ T Cells.

In preliminary assays culturing peripheral PBMCs with crude Acai we identified γδ T cell activation (up-regulation of CD69/IL-2Rα expression). This activity was independent of prenyl phosphates since bovine γδ T cells responded to this extract, suggesting the agonist activity could be due to polyphenols and/or polysaccharides (data not shown). As a first step toward identification of the activating component(s) of the Acai extract, we separated the crude extract via Kupchan fractionation[370] or EtOH precipitation. Dose response assays were performed on the various fractions by measuring bovine γδ T cells activation in PBMC cultures. As shown in Figure 5.1A, EtOH-precipitated material induced γδ T cell activation as measured by IL-2Rα expression at low ng/mL concentrations. Furthermore, no activity was detected in the EtOH-soluble or non-polar fractions. Since polysaccharides precipitate in EtOH whereas polyphenols, in general, remain soluble in ethanol, this suggested that polysaccharides were the agonist fraction in Acai. We next compared LAL reactivity in the EtOH-precipitated Acai to Yamoa-

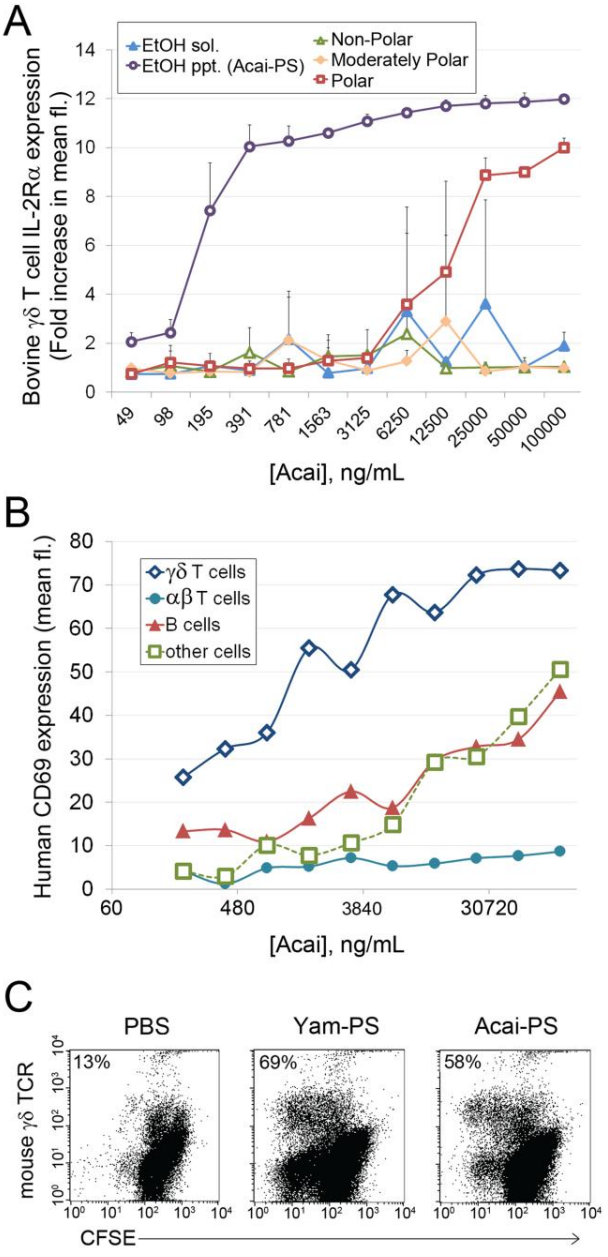
derived polysaccharides (Yam-1) which are described in a previous report.[4] EtOH-precipitated Acai had far less potential endotoxin reactivity than Yam-1 (>0.0000125EU/ng for Acai versus 0.194EU/ng for Yam-1). These experiments indicated that Acai polysaccharides activate γδ T cell populations without the potential endotoxin contamination common to Yamoa and other polysaccharides.

To determine if Acai polysaccharides similarly activate mouse and human cells, we further purified the polysaccharides from EtOH-precipitated Acai using DEAE cellulose. This fraction, Acai-PS, was 92.1% polysaccharides as determined by phenol-sulfuric acid assay, and as expected, retained low LAL reactivity (0.0004EU/ng). As shown in Figure 5.1B, Acai-PS increased the expression of CD69 on human lymphocytes, with the most robust activity detected in γδ T cells. Other cell populations including B cells and the unstained gate (other cells; presumably $CD3^{dim}$ T cells, $CD19^{dim}$ B cells, NK cells, and/or small monocytes) demonstrated increased CD69 expression to some degree. Mouse γδ T cells also responded to the Acai-PS fraction, as demonstrated by increased proliferation in response to rmIL-2 (Figure 5.1C). This *in vitro* priming response was repeated in $TLR2^{-/-}$ and wildtype mice (C57BL/6). Importantly, we observed no toxic effects from Acai *in vitro* at concentrations up to 500μg/mL (Human PBMCs, data not shown) indicating these observed γδ T cell responses were not a result of cellular distress.

## γδ T Cell Agonist Activity in Acai was Observed in the Polysaccharide and not the Polyphenol Fraction

Since earlier reports demonstrate immunomodulatory activity of Acai polyphenols[365-367] and we have found that some polyphenols are potent γδ T cell agonists,[1,2] we purified polyphenols from Acai and tested them for activity. As shown in Figure 5.2A, purified

Figure 5.1 γδ T cell Stimulatory Activity in Acai is Concentrated in the Polysaccharide Fraction and Effective in all Species Tested.



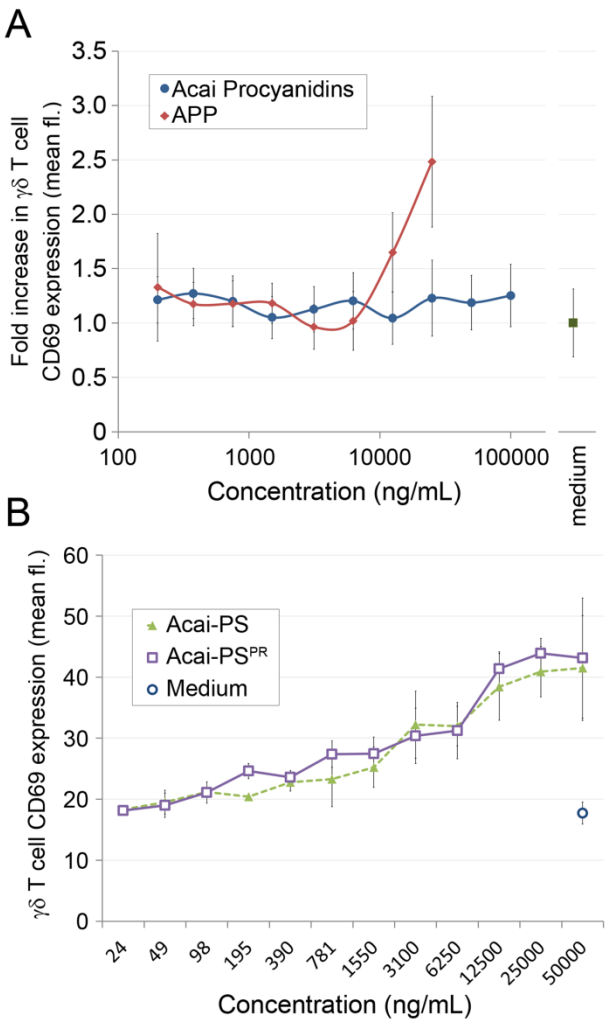

A) Aqueous extract of Acai was separated via EtOH precipitation or Kupchan fractionation. The resulting fractions were lyophilized, re-suspended in water, and tested in bovine PBMC culture for γδ T cell agonist activity. Data represent mean and SD from triplicate cultures from the same calf. EtOH precipitant (ppt.) responses are representative of cultures from three calves and three separate preparations. B) Human cells were cultured for 48h with Acai-PS or medium prior to analysis for cell activation (CD69 expression) using flow cytometry. Values represent the average response of duplicate cultures from a single donor. Data are representative of two experiments. C) CFSE-labeled TCRα$^{-/-}$ splenocytes were cultured in X-VIVO with PBS, YamPS (9μg/mL), or AcaiPS (10μg/mL) for 24h, then medium was replaced with fresh medium containing IL-2 and cultured for an additional 72h. Percent γδ T cell proliferation is given for each scatter plot, and representative of two AcaiPS preparations.

Acai polyphenols did not increase CD69 expression on γδ T cells at dosages nearly ten times higher than the polyphenol agonist, Apple Polyphenol (APP) [1]. Also, there was no shift in the dose response curve of AcaiPS in which polyphenols were removed ($AcaiPS^{PR}$) by PVPP pre-treatment (Figure 5.2B). These results indicate that the previous reports describing polyphenol-induced immune responses were not a result of activated γδ T cells.

To better characterize the Acai polysaccharide agonist, the Acai-PS extract was then fractionated by preparative Sepharose 6B size-exclusion chromatography to obtain three fractions, which were selected based on the total carbohydrate elution profile (designated as: Acai-1, Acai-2, and Acai-3, Figure 5.3A). Based on calibration curves derived from pullulan standards,[8] we determined that fraction Acai-1 was composed of molecules with mass ~200,000Da and a small sub-peak at ~800,000Da, which could represent molecular aggregates. Acai-2 had a broad peak at ~26,000-60,000Da, and Acai-3 contained a broad peak at 4,000-12,000Da (Table 5.1). As expected, all of these fractions remained low in LAL reactivity (Table 5.1). The fractions were then analyzed for polysaccharide and protein content and found to contain >99% carbohydrate and <1.0% protein (Table 5.1). Note that the carbohydrate profile was in accord with that of refractive index chromatogram obtained by HP-SEC (Figure 5.3B).

All three fractions exhibited fluorescence emission in the broad region of 370-540nm (Figure 5.3C). This finding suggested the presence of aromatic groups in context of the polysaccharides; however, this method will detect minute levels of aromatic groups, therefore the relative contribution of aromatics could not be estimated from this assay.

Figure 5.2 Acai Fruit does not contain Polyphenol-based γδ T Cell Agonists.

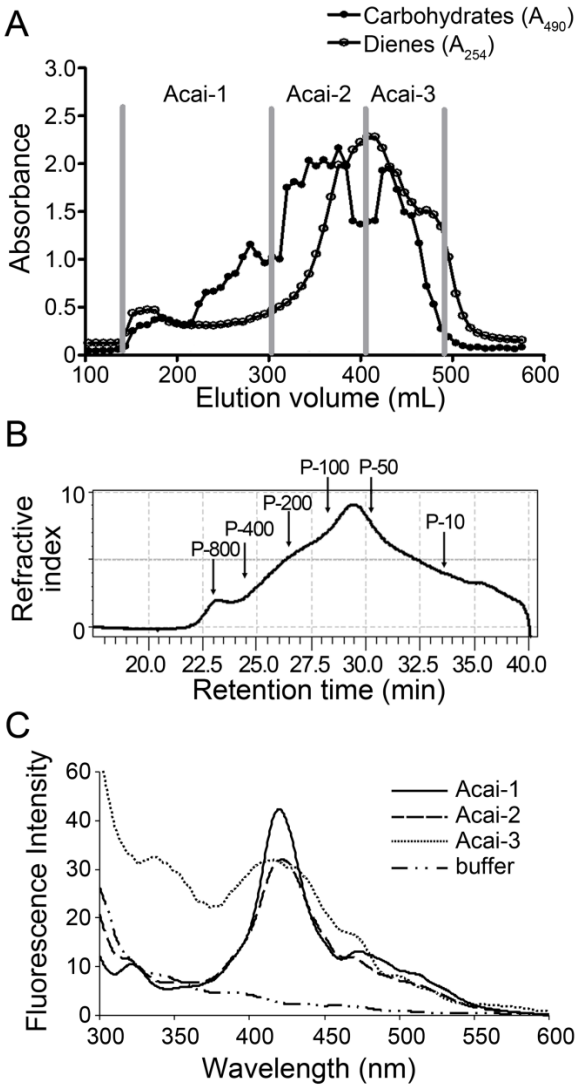



A) PVPP-extracted Acai polyphenols were cultured with human PBMCs to detect γδ T cell activation. As a control, APP was used to induce polyphenol-based γδ T cell activation. B) Acai-PS was treated with PVPP to remove polyphenols and the resulting preparation (Acai–PS$^{PR}$) or untreated Acai-PS was cultured with human PBMCs. γδ T cell activation from the subsequent cultures was measured by FACS as induced CD69 expression. Results are from three individual donors. Error bars represent SD. Experiments were performed independently with respect to donor, experiment date, and Acai-PS$^{PR}$ extraction.

To estimate potential aromatics, we performed a Folin-Ciocalteu assay. Results indicated polyphenols were a trivial component of the Acai-1 fraction (0.2%) and a minor component of the Acai-2 and Acai-3 fractions, 2.8% and 5.9%, respectively (Table 5.1). This result further demonstrated that the polysaccharide and not the polyphenol fraction was the predominant source of γδ T cell activity in the Acai preparation.

Very-high-field (600 MHz) $^{1}$H NMR was used to characterize the structure of the native Acai polysaccharides. The spectra from all three fractions (Supplemental Figure S5.1) suggested a backbone structure resembling native arabinogalactans isolated from various plant sources.[383,384] Using evaluation methods previously described for the sugar composition of the arabino-3,6-galactans (type II),[383,384] we identified the presence of unsaturated α-rhamnopyranose, β-galactopyranose, α-arabinofuranose, and α-galacturonopyranose. All spectra also indicated a significant amount of *N*- and *O*-acetyl (1.9–2.0ppm), methyl (0.75 and 1.1ppm), and alkylamide (3.21ppm) groups.
Type II arabinogalactan contains a β-(1,3)-linked galactan backbone with side chains containing arabinose and galactose residues and has reported biological activities in other systems,[261,385] but importantly, polysaccharide fractions containing type II arabinogalactan also contain the most activity as γδ T cell agonists.[4,8] To evaluate the Acai polysaccharide fractions for arabinogalactan, the Yariv test was performed. All three fractions resulted in a positive reaction, indicating they contained arabinogalactan type II (Table 5.1). Sugar composition analysis revealed that the Acai polysaccharides consisted primarily of arabinose, galacturonic acid, and galactose (Table 5.2).

Figure 5.3 Chromatographic Characterization and Fractionation of Acai Polysaccharides.




Water extract of Acai was prepared and separated on DEAE-cellulose column (Acai-PS) and quantified using multiple methods: A) Acai-PS fractionation by gel chromatography on Sepharose-6B column. Three polysaccharide fractions (designated Acai-1, Acai-2, and Acai-3) were selected based on total carbohydrate and diene conjugate contents. B) High pressure gel filtration chromatography elution profile of Acai-PS with a refractive index detector. C) Synchronous spectra of fluorescence polysaccharides, isolated from Acai-PS. 500μg/ml of polysaccharide fraction in $NaHCO_3$ buffer (pH 8.5).

Table 5.1 Biochemical and Spectral Properties of Acai Polysaccharide Fractions

| Polysaccharide fraction | Average molecular weight (kDa) | potential endotoxin (ng/µg) | Protein content (w/w) | Total phenolic content (w/w) | Type II arabinogalactan |
|---|---|---|---|---|---|
| Acai-1 | 200 | 0.33 | 0.35% | 0.2% | Positive |
| Acai-2 | 26-60 | 0.05 | 0.43% | 2.8% | Positive |
| Acai-3 | 4-12 | 0.01 | 0.60% | 5.9% | Positive |

Table 5.2 Monosaccharide Composition of Acai Polysaccharide Fractions

| | Polysaccharide fraction | | |
|---|---|---|---|
| Monosaccharide | Acai-1 | Acai-2 | Acai-3 |
| Fucose | 0.5 | 0.6 | 1.3 |
| Rhamnose | 4.5 | 4.1 | 4.9 |
| Arabinose | 47.0 | 26.2 | 18.8 |
| Glucosamine | N.D. | N.D. | N.D. |
| Galactose | 11.5 | 17.9 | 16.5 |
| Glucose | 2.3 | 10.4 | 18.8 |
| Lyxose | N.D. | N.D. | N.D. |
| Mannose | N.D. | 4.7 | 8.4 |
| Xylose | 2.8 | 9.7 | 8.0 |
| Galacturonic acid | 28.4 | 24.7 | 21.7 |
| Glucuronic acid | 3.0 | 1.8 | 1.5 |

The data are present as mol% for each sugar. Individual components were identified and quantified based on elution of known standards. N.D. – not detected

These results indicated that the Acai polysaccharide fractions Acai-1, Acai-2, Acai-3 contain different structures as determined by size, sugar content, and NMR. Furthermore, these fractions were predominantly polysaccharide with very low polyphenol or protein content.

Acai Polysaccharides Induce γδ T
Cell and Myeloid Cell Activation.

The activity of the Acai-PS fractions were then tested using the CD69 expression assay for human PBMC cultures. As shown in Figure 5.4A, treatment with Acai-1 and to a lesser extent Acai-2 activated human γδ T cells with minimal activity on αβ T cells and B cells. We also tested the monomeric sugars from Acai-1. These sugars had no agonist effect (data not shown), indicating the complex structure of Acai-PS is important to its immune activity.

Since other polysaccharide preparations are potent inducers of reactive oxygen species formation,[8] we tested Acai polysaccharides for similar responses. In the absence of any treatment, human PBMCs did not generate detectable ROS (Figure 5.4B, control), whereas the addition of Acai-PS fractions activated ROS production with a lag-phase of around 30min. A concentration-dependent enhancement of ROS production was observed in PBMCs treated with 37.5-300μg/mL of each polysaccharide fraction (Figure 5.4C). As in the γδ T cell activation assays, Acai-1 demonstrated the most activity.

We next examined cytokine production in Acai-treated human PBMCs. Among the twelve cytokines analyzed, six were consistently induced in PBMCs with 100μg/mL of Acai polysaccharide fractions, as compared with control cells. For Acai-1, these included IL-1α [fold increase (FI) = 4.8], IL-1β (FI = 15.9), IL-6 (FI = 223), IL-10 (FI = 57), TNFα (FI = 23), GM-CSF (FI = 4.2) (Figure 5.4D).

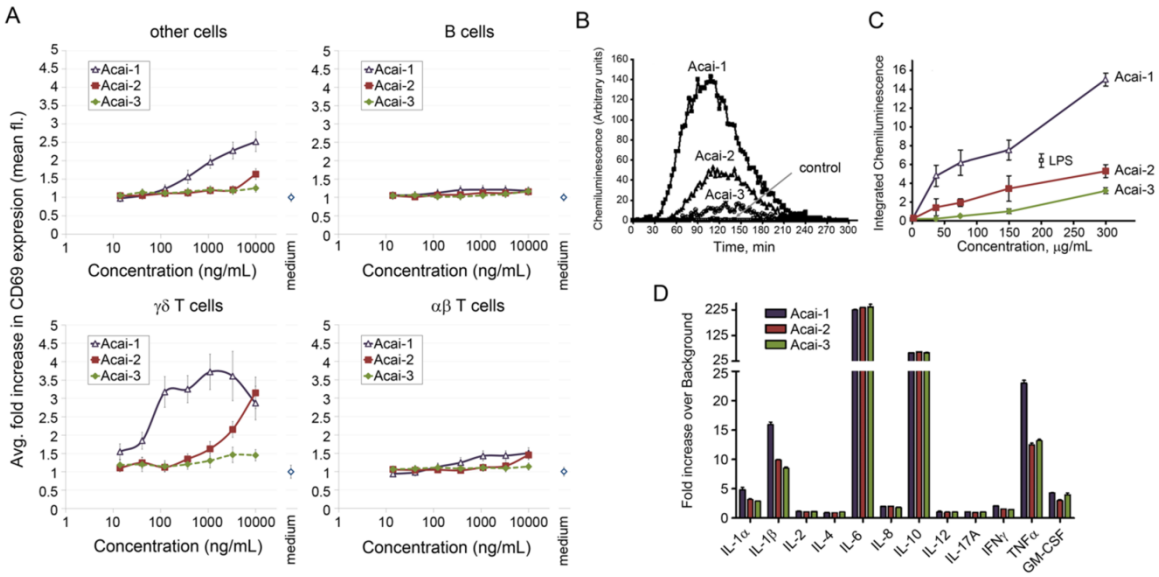
The ROS formation and cytokine secretion profile indicated that Acai polysaccharides function similarly to polysaccharide preparations active on monocytes.[4,8]

Therefore, we further analyzed the monocyte cell response to Acai polysaccharides. Although the amount of endotoxin (LPS) was very low and far larger amounts are required to activate γδ T cells,[4,111] monocytes are exquisitely sensitive to endotoxin. Thus, further steps were taken to ensure that endotoxin was not a component of the Acai preparation. To this end, we prepared a detoxified Acai-1 by elution through a column of endotoxin-removing gel (denoted Acai-$1^{ER}$). To quantify dose-dependent effects of Acai polysaccharides on monocyte-associated cytokine production, levels of TNFα and IL-6 were determined in cells treated with the polysaccharide fractions by ELISA. As shown in Figure 5.5A, incubation of PBMCs with the fractions enhanced TNFα and IL-6 production in a dose-dependent manner. Acai-1 and Acai-$1^{ER}$ induced very similar responses indicating the minor endotoxin contamination had no effect on the activity of Acai-1. Furthermore, as with previous experiments, the Acai-1 fractions were the most active fraction at low concentrations (<1μg/mL). Although a slight decrease in TNFα production in Acai-1-treated cultures from 5 to 10μg/mL was noted, the level of the cytokine was increased when cells were exposed to a higher dose (100μg/mL) of Acai-1. We hypothesized this was due to the inherent heterogeneous nature of cell populations in PBMC cell preparations and their differing responses to the polysaccharides.

To address monocyte-specific responses to Acai polysaccharides, we tested the fractions on a human monocyte cell line, MonoMac-6. As with the PBMC cultures, Acai-$1^{ER}$ induced nearly identical responses to the non-endotoxin-cleared Acai-1 in MonoMac-6 cells (Figure 5.5A). MonoMac-6 cells also responded similarly to human

PBMCs (Figure 5.5B) without the cytokine secretion plateau, confirming monocytes respond, in a dose-dependent manner, to Acai polysaccharide culture. The elimination

Figure 5.4 Acai Fractions Induce Cell Activation as well as ROS and Cytokine Production.




A) PBMCs were collected from three donors and cultured with indicated agonists at various concentrations (x axis). Cultures were performed in triplicate. Data represent the mean fold increase (CD69 mean fluorescence) versus medium for each agonist/concentration value. Error bars represent normalized SD. B) PBMCs were incubated with polysaccharide fractions (150µg/mL) and ROS production was measured over 300min. C) ROS production from PBMCs was measured as a function of dose. PBMCs were incubated with the indicated concentrations of polysaccharide fractions, LPS, or vehicle only for 24h. ROS production was then measured for 3h from triplicate samples. Data represent the mean ± S.D. total luminescence over 3h. Values are from one experiment, representative of three independent experiments. D). An ELISA was used to measure cytokine production by human PBMCs treated with 50µg Acai-PS. Values represent the mean fold increase versus medium control cultures from triplicate wells. Error bars represent SD. Cultures were from one subject. Production of IL-1β, IL-6, GM-CSF, and TNFα, as well as limited IL-8, was confirmed in PBMCs from at least one additional donor using different ELISA reagents.

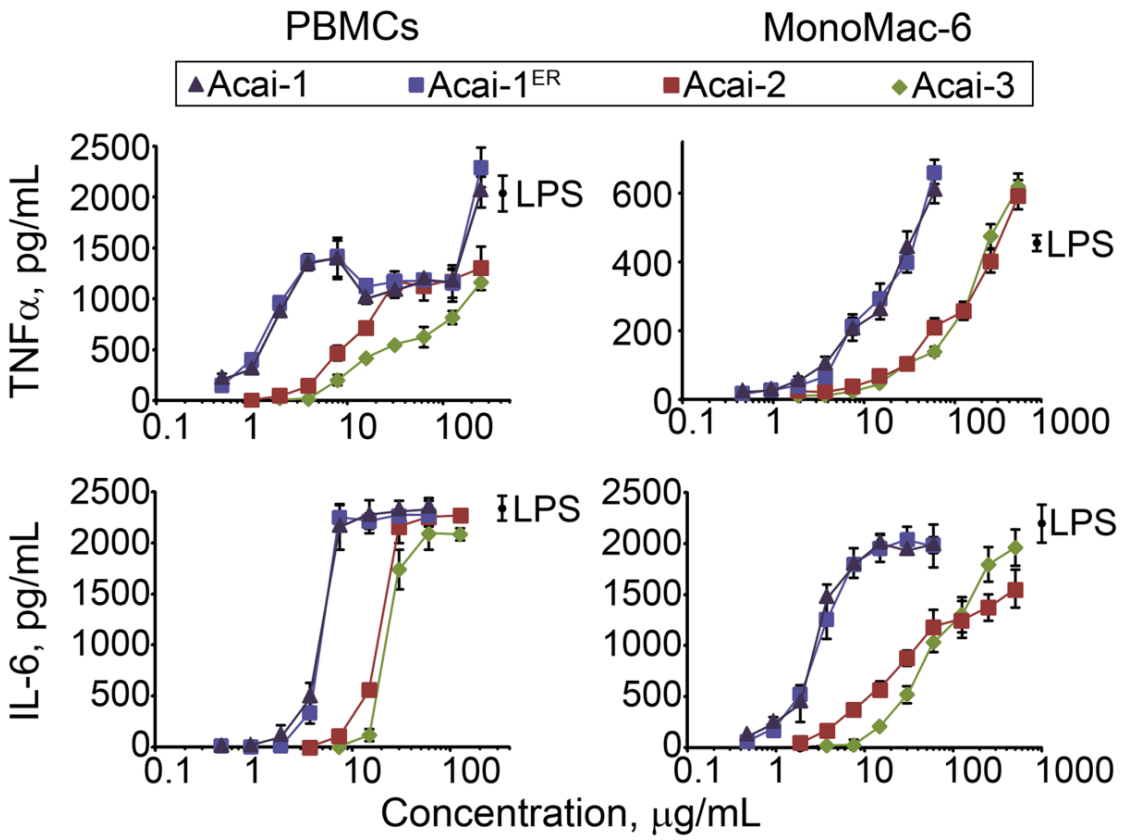
of the cytokine secretion plateau and the greatly reduced TNFα production in the MonoMac-6 cultures indicated monocytes were not the sole responding factor to Acai polysaccharides. These results, in combination with the activation of γδ T cells, were in accord with our earlier studies on Yamoa that indicate γδ T cells, monocytes, and possibly other cells cooperate to induce innate responses to polysaccharides.[4]

## Acai Polysaccharides Induce Immune Cell Recruitment and Activation Responses *in vivo*

Since Acai polysaccharides affect multiple cell types, we next sought to examine the combined effects of Acai-derived polysaccharides *in vivo*. To this end, we first examined their effect on the recruitment of neutrophils after intraperitoneal (i.p.) injection in mice. As shown in Figure 5.6A, Acai-PS induced neutrophil recruitment into the peritoneum, similar to $LAL^{+}$ polysaccharides, Yam-1. Consistent with our previous report demonstrating a clear difference between Yam-1- and LPS- induced responses,[4] the effect was not contingent upon on MyD88 signaling (Figure 5.6B). Since $MyD88^{-/-}$ mice are documented to possess an atypical immune response,[386,387] no conclusions can be reliably drawn from the apparent reduction of peritonitis in $MyD88^{-/-}$ mice versus wildtype mice; it may be that there is a MyD88-dependent component to the full response or it may be due strain differences. These results indicate that the *in vitro* immunostimulatory responses we observed toward Acai polysaccharides were preserved *in vivo*.

To determine if Acai-derived polysaccharides induce immune responses at mucosal surfaces, mice were treated i.t. with 500µg Acai-PS and, 24hrs later, cells in the BALF and lung tissue were extracted to measure myeloid cell activation/recruitment. In the

Figure 5.5 Effect of Acai Polysaccharide on TNFα and IL-6 Production in MonoMac-6 and Human PBMCs.



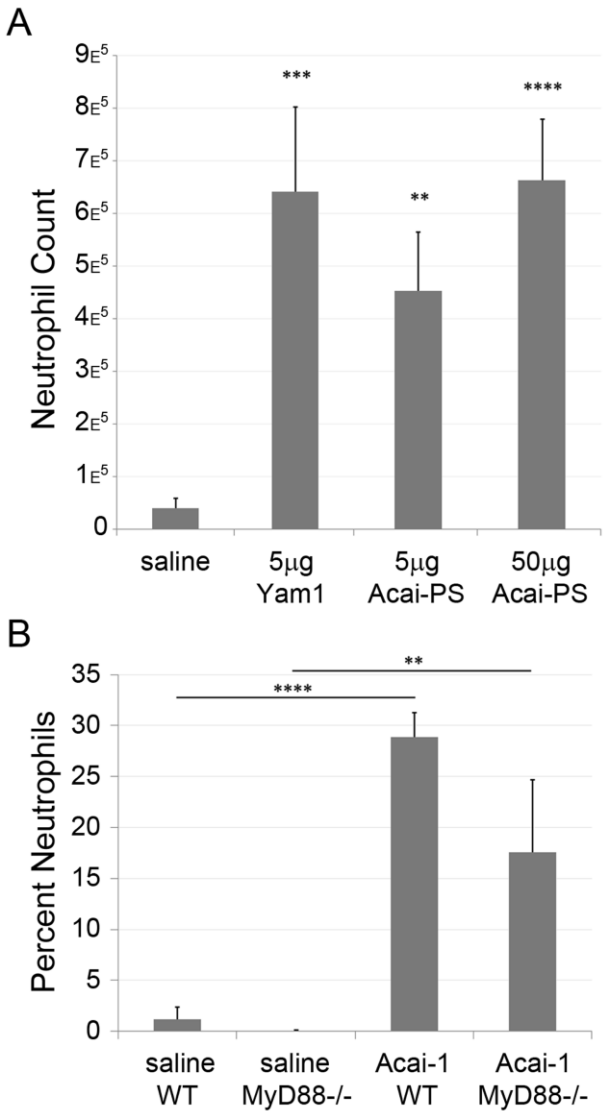

Human PBMCs or MonoMac-6 macrophages were incubated for 24h with the indicated concentrations of polysaccharide fractions Acai-1, Acai-1 pretreated with endotoxin-removing gel (Acai-$1^{ER}$), Acai-2, Acai-3, or 200ng/mL LPS. Cell-free supernatants were collected, and extracellular TNFα and IL-6 were quantified by ELISA. Values represent the mean ± S.D. of triplicate samples from one experiment, which is representative of at least three independent experiments.

BALF, alveolar macrophages (autofluorescent, Oval gate) increased expression of CD11c (Figure 5.7A), indicating these resident cells were activated. Similar to the peritonitis experiments, there was also a neutrophil influx detected in the BALF as shown by the

increased $CD11b^{+}/CD11c^{-}$ population (Figure 5.7A). These cells were confirmed to be neutrophils by Gr-1 expression and their distinctive light scatter profiles (data not shown). The remaining lung tissue was then homogenized and collagenase digested to collect the lung interstitial population. Flow cytometry detected an additional CD11b/CD11c positive cell population (Figure 5.7B, rectangles). It is unknown from these experiments whether these additional myeloid cells were recruited or whether they were activated resident cells. Regardless of the source of these activated myeloid cells, these experiments demonstrate a change in lung innate immune cell profile upon Acai administration.

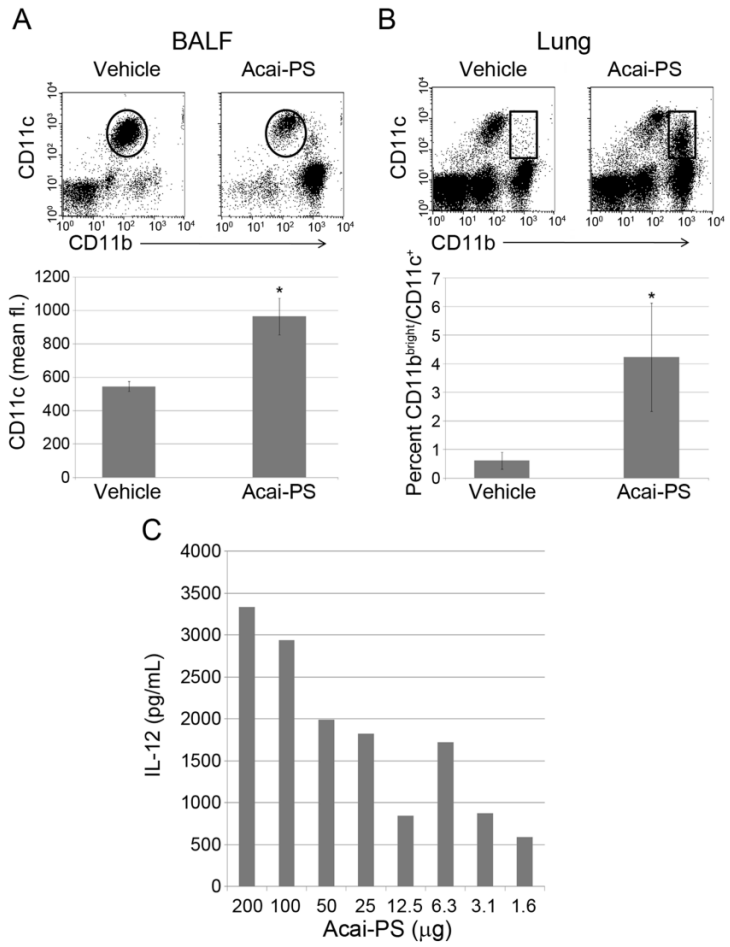
To further characterize the lung response to Acai, the BALF was tested for the proinflammatory cytokine, IL-12. In initial studies from four mouse strains (BALB/c, C57BL/6, C3H, and $MyD88^{-/-}$) we observed an increased IL-12 content in Acai-PS-treated versus control animals ($n \geq 2$ for each strain, data not shown). Therefore, to estimate the dose response, BALB/c mice were treated i.t. with a range of Acai-PS doses and the concentration of IL-12 in the BALF was measured. As shown in Figure 5.7C, Acai-PS dose-dependently induced IL-12 production within the lung, indicating a proinflammatory Th1 response. Thus, the myeloid cell-associated response to Acai polysaccharides was conserved in both lung and peritoneal tissues. In contrast to the current thought that Acai polyphenols are responsible for immune enhancement,[366,367,388] these data demonstrate immunostimulatory properties of Acai polysaccharides both *in vitro* and *in vivo*.

Figure 5.6 Acai Polysaccharides Induce MyD88-Independent Neutrophil Influx to the Peritoneum




A) BALB/c mice were injected intraperitoneally with saline, Acai, or Yam-1. After 4h, mice were euthanized, peritoneal cells collected, and total neutrophil counts measured by flow cytometry. Data represent the average total cell count from a minimum of four mice per treatment group and error bars represent the SEM. B) C57BL/6 or MyD88$^{-/-}$ mice of mixed ages (12-23 weeks) and sexes were injected i.p with Acai-PS (400μg) or saline and neutrophil flux was measured as in A) without the use of FACSbeads to estimate total cell counts. The data are representative of the mean percentage of neutrophils in the wash +/- SD from a single experiment with 3-4 mice/group. p-values (Student's T test) for both figures are represented as: *<0.05, **<0.01, ***<0.005, ****<0.001

Figure 5.7 Intratracheal (i.t.) Treatment with Acai-PS Activates Lung Myeloid Cells and Induces IL-12 Production in Mice.




BALB/c mice (n=3) were treated i.t. with vehicle ($dH_20$) or 500µg Acai-PS in a volume of 100µL. BALF and lung cells were isolated 24h post-treatment. Cells were stained with antibodies for CD11b and CD11c and analyzed via flow cytometry for myeloid cell activation/recruitment. A) BALF alveolar macrophages were gated (autofluorescent/$CD11c^+$; ovals) and activation was measured as an increase in mean CD11c-associated fluorescence within this gate. B) Cells in the lung interstitium were collected via collagenase extraction and similarly analyzed by FACS for myeloid cell recruitment/activation. The percentage of myeloid cells (rectangle gate) in relation to total live leukocytes was compared between Acai-PS and vehicle treated mice. Data from A) and B) are representative of three similar experiments and were repeated in C57BL/6 (3 experiments) and C3H/HeOuJ (2 experiments). C) BALF was collected from BALB/c mice provided varying dosages of Acai-PS i.t. Cells were removed by centrifugation, and IL-12(p70) concentration was determined in the supernatant fluid by cytokine ELISA.

## Discussion

Herein, we show that polysaccharides, but not polyphenols, derived from Acai fruit pulp have potent immunomodulatory activity and stimulated both γδ T cells and myeloid cells. In addition, other cell populations were shown to upregulate CD69 expression. Although not directly assayed, these cells were within in the lymphocyte gate, did not express T cell or B cell markers, and thus are presumed to be NK cells. Although Acai is heavily marketed and currently taken by the general public to enhance immune cell function (presumably through antioxidant function), for weight loss, and for a variety of other unfounded claims, there have been few studies on its specific mechanisms of action. Indeed, much of the information justifying its use is anecdotal. The *in vitro* response to Acai-PS was conserved between mouse, bovine, and human cells and correlated with *in vivo* responses. In mice, Acai-PS incited neutrophil recruitment to the peritoneum and lung, as well as activated DCs/macrophages in the lung. This peritonitis response occurred independent of MyD88 signaling. This latter result, in combination with the minimal LAL reactivity, shows that bacterial-derived endotoxin is not responsible for the activity of Acai-derived polysaccharides and that these polysaccharides contain a distinct innate immune agonist.

The limited analyses reported to date suggest that polyphenols represent the immunomodulatory compounds in Acai.[365,366,368,369] In contrast, the activity reported here tracked solely to the polysaccharide fraction of the Acai fruit pulp and we found little to no activity from the polyphenols within this extract. Evidence against polyphenol-

induced γδ T cell activation was based on: 1) an absence of immune cell activity in polyphenols purified from Acai-extract (Figure 5.2A), 2) a retained bioactivity in Acai preparations depleted of polyphenols (Figure 5.2B), and 3) the limited amounts of polyphenols in the purified Acai-fractions (Table I). It is unlikely that the very small amounts of free polyphenols not removed by PVPP could account for our results, since a defining characteristic of γδ T cell immunomodulatory polyphenols is the relatively high concentrations (low μg/mL) required to induce cellular responses *in vitro*.[1,2] It remains possible that polyphenol-complexed polysaccharides are required for biological activity since there were a small amount of polyphenols in the Acai-1, Acai-2 and Acai-3 fractions. However, the most active fraction, Acai-1, had the least amount of polyphenol (0.2%, Table 5.2) rendering this theory unlikely. It is therefore likely that the previous reports describing polyphenol activity were a result of general antioxidant effects. This is not unexpected since Acai has a very high antioxidant capacity.[367,389]

The fractions tested herein were derived from the Acai fruit pulp since it is the primary source of nutritional supplements and foodstuffs. The fruit pulp contains a relatively low concentration of the preeminent γδ T cell polyphenol agonist, OPCs,[388,389] which could explain the lack of γδ T cell agonist activity. However, the seed from Acai fruit contains an enriched OPC profile very similar to APP or grape seed.[369] Since OPCs from APP, grape seed, and others activate γδ T cells,[1,2,390] polyphenols extracted from Acai seeds may have similar effects on γδ T cells. While identifying the potential γδ T cell agonist activity of the polyphenol extract from Acai seed was beyond the scope of

these studies, additional studies are underway to determine its biological activity. If it holds true that Acai seed polyphenols contain γδ T cell agonist activity, the seed could be prepared as a distinct nutritional supplement. Acai seeds are currently a byproduct of the Acai fruit, and are generally wasted or being used as pig feed or potting soil.[369] This seed may be an alternative and economically feasible source for γδ T cell-activating polyphenols for human applications.

Questions have been raised about the role of microbial contaminants in plant-derived products contributing to immunomodulatory activity[391,392] thereby necessitating strict control over potential contaminants. Furthermore, products such as polysaccharides are particularly difficult to control for since they can cause false-positive LAL reactions[393,394] and are difficult to isolate from endotoxin using conventional methods.[4] We found no evidence of microbial contamination in the Acai extract, as evidenced by minimal LAL reactivity and negative results of attempted bacterial culture (data not shown). Furthermore, the bioactivity in Acai-1 could not be removed by polymyxin B (Figure 5.5), and activity occurred in animals deficient in MyD88 signaling (Figure 5.6B). Thus, sensing of microbial products through TLRs likely does not account for the innate cell responses shown in this report. However, it could very well be that MyD88-independent, yet TLR-dependent, pathways, such as TLR4-mediated TRIF signaling, are involved in recognition of the polysaccharides and these issues are currently under study. Furthermore, elaboration of processed IL-1β suggests that Acai polysaccharides could affect the inflammasome, for which agonists are highly variable.[332,395] This possibility clearly warrants, and is under, further investigation.

To date, we have defined myeloid cell agonist activity in a number of plant extracts, including extracts from juniper berries,[8] Artemisia,[7] prickly-pear cactus,[5] Yamoa,[4] and now Acai. Activity on γδ T cells has also been observed with these extracts, excluding cactus (Holderness, unpublished results).[4] Thus, the relevant γδ T cell agonists may be polysaccharides common to many plants. As such, we predict that other plants contain bioactivity similar to that in Acai, which may account for the expansion of γδ T cells in people that have consumed certain fruit and vegetable extracts.[364]

As discussed in Graff *et al*., Yamoa is purported to be beneficial in asthma.[4] γδ T cells can play a prominent role in mediating asthma responses. In mice, lung γδ T cells are present that can either promote or restrict asthma-like symptoms.[396] Asthma is a Th2-mediated disease in these cytokine responses. Asthmatics contain more lung γδ T cells than non-asthmatics[397] and this γδ T cell population is largely responsible for the production of inflammatory Th2 cytokines during allergen challenge.[398] Therapies to increase Th1 responses can be effective in alleviating symptoms.[399] We originally proposed that the plant polysaccharides we characterized from Yamoa, and now Acai, might tip the balance in the lung towards a Th1 response, but we had no direct evidence in support of this hypothesis. Here we found that Acai-1 directly induced IL-12 production in the mouse lung. IL-12 release favors a downstream Th1 response via IFNγ production from leukocytes in the mucosa.[400] Thus, we provide, for the first time, mechanistic evidence for the potential benefit of some plant polysaccharides by driving Th1 responses in the lung. In addition, IFNγ is crucial for host defense responses against

intracellular bacterial pathogens of the lung, such as *Francisella tularensis*[401] and *Coxiella burnetii*.[402] Efforts are currently underway to test the effectiveness of Acai polysaccharides in countering lung infections in these and a variety of other pulmonary infection models.

Another issue currently under investigation is whether the plant polysaccharide-induced immune cell activity can also be produced following oral ingestion. There are many variables within these experiments such as the effects of gastric enzymes, low pH, normal bacterial flora, and agonists in a normal diet on the agonist activity. However, Acai polysaccharide extracts are certainly resistant to harsh chemical conditions similar to the stomach, including high heat (boiling; see preparation in materials and methods) and low pH (1M HCl; data not shown). To date, it is uncertain whether Acai polysaccharides translocate across epithelial barriers; however, there is precedence for modulation of systemic immune activity by consumption of plant- and microbial-derived glucans[403] as well as large polysaccharide polymers from *Aureobasidium pullulans*[404] and *Ganoderma lucidum.*[262,405] Furthermore, immunomodulatory polysaccharides can impact intestinal leukocytes and enterocytes,[406] in the event that the polysaccharides described herein are restricted to acting upon cells within the intestine, intraepithelial γδ T cells could still be targeted. Potential therapeutic applications for a gut-restricted γδ T cell agonist include epithelial healing[134,358] and improved immune responses to a variety of pathogen-associated diseases.[350] In preliminary studies, mice administered Acai-PS or Yam-1 by oral gavage produced a cytokine response that could be detected within the serum (Holderness and Hedges, unpublished observations). Therefore, it would seem

that Acai polysaccharides are capable of inducing a systemic immune response. These preliminary results are being investigated in detail as part of ongoing studies to determine the *in vivo* and therapeutic potential for γδ T cell-agonist polysaccharides.

In closing, characterization of the immune responses or lack thereof for common nutritional supplements is important for potentially isolating new drug candidates but also for preventing potential misuse by the public. Herein, we define potent immunomodulatory activity from Acai on monocyte and γδ T cell populations. Unlike previous reports describing activity in the polyphenol fraction, we instead identified activity in the polysaccharide fraction. These polysaccharides were able to induce cell recruitment and Th1 responses *in vivo*. As such, potential applications for these polysaccharides include asthma and infectious disease. The absence of significant LAL reactivity is critical for the description of polysaccharide-induced immune responses *in vivo* and provides a readily available source for the development of a clinical preparation.

## Acknowledgements

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# CHAPTER SIX

# ACAI POLYSACCHARIDES ACTIVATE THE INNATE IMMUNE SYSTEM VIA COMBINED TLR4 AND DECTIN-1 ACTIVATION

## Contribution of Author and Co-Authors

Manuscript in Chapter Six

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Contributions: Designed experiments and obtained funding.

Manuscript Information Page

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## Abstract

Recognition of non-self polysaccharides by Pattern Recognition Receptors (PRR) of the innate immune system leads to the activation of inflammatory responses that are critical to the protection against different pathogens. There are numerous PRR that are able to recognize polysaccharide structures and thus differently or cooperatively mediate innate responses. Recently, we described the immunostimulatory properties of a polysaccharide from the fruit of the Acai palm. This polysaccharide preparation activates monocytes, NK cells, and γδ T cells to generate Th1 responses in the lung and provides protection against *Francisella* challenge. This report characterizes the PRR stimulated by this polysaccharide. Specifically, we demonstrate that Acai polysaccharide activated innate immunity through the unique ability to stimulate both TLR4- and Dectin-1-mediated responses. TLR4 responses were ablated when the acetyl groups of the polysaccharide were removed, suggesting a similar active structure to that of *Klebsiella* capsule polysaccharide. Furthermore, this polysaccharide bound Dectin-1 via β-glucan structures to generate ROS in a TLR4-independent fashion. Together, these responses robustly activated the mouse and the human innate immune system and may explain the dynamic *in vivo* phenotype induced by these polysaccharides.


## Introduction

As part of the surveillance program for pathogens, the innate immune system recognizes many different pathogen moieties through pattern recognition receptors

(PRR). In terms of polysaccharides, the predominant PRR are the Toll-like receptors 2 and 4, as well as the polysaccharide receptors, Dectin-1, Dectin-2, Macrophage Mannose Receptors (MMR; CD206, CD280), DC-SIGN (CD209), Mac-1(CD11b/CD18). These receptors form a complex system for recognizing polysaccharides and initiating appropriate innate immune responses. An optimal response to polysaccharide requires both receptor-mediated signaling and endocytosis.

Dectin-1 is a C-type-like lectin that is able to recognize fungal, bacterial, and plant polysaccharides via β-1,3 glucan linkages.[21,407,408] The signaling responses to Dectin-1 are numerous, it can mediate endocytosis through Mac-1 and probably other receptors,[72] syk/CARD9 signaling and thereby, NFκB activation,[75] Raf-1 activation,[75] and canonical and non-canonical inflammasome activation.[79] Many of these responses are dependent upon endocytosis[78] but others are not.[79] Depending upon the components of Dectin-1 signaling that are activated, and the co-receptors involved, Dectin-1 mediates numerous inflammatory responses including cytokine production via NFκB, ROS production via syk, modulating TLR responses, and the production of mature IL-1β.[79,332]

TLR4 signaling is likewise highly dependent upon co-receptors and endocytosis for mediating ligand recognition and signaling pathways. Although TLR4 is dispensable for many fungal polysaccharide-mediated responses, there are examples of plant, fungal, and bacterial polysaccharides engaging this receptor.[267,271,273,274,277,278,409] In *Klebsiella* capsule polysaccharide, this recognition is mediated by acetylation and/or pyruvation of the saccharide residues.[279] Unlike other TIR-containing receptors, TLR4 can use both adaptors, MyD88 and TRIF. The preferential signaling of these molecules is regulated at

many levels including endocytosis. During TLR4 ligand engagement at the surface, responses occur primarily via the MyD88 pathway. When endocytosed, these cells alter their signaling pathways to incorporate the TRIF pathway, leading to the additional production of type I IFN via IRF3.[93] Recently, Zanoni *et al*. demonstrated that TLR4 endocytosis is mediated, at least in part, via the TLR4 co-receptor CD14 and the C-type lectin-associated-kinase syk.[61]

Plant polysaccharides are able to activate many of the signaling pathways attributed to their fungal counterparts. There are reports showing that they require the use of TLR4, CD14, and Mac-1, but not Dectin-1. Previously we reported that an endotoxin-free, acetylated and pyruvated plant polysaccharide from Acai (AcaiPS) activated innate immune cells to generate a response similar to TLR agonists.[9] This polysaccharide activates monocytes and γδ T cells *in vitro*. Intratracheal administrations lead to lung remodeling and IL-12 production. In a follow-up report, we tested the ability of this Th1 response to protect against intracellular bacterial challenge with *Francisella tularensis*. AcaiPS generated a Th1-centric response characterized by IFNγ production in NK and γδ T cells of the lung. This IFNγ production by AcaiPS was required for, and protected mice against, *F. tularensis* and *Burkholderia pseudomallei* challenge.

To determine if innate immune recognition of AcaiPS could be attributed to the pathways described for other stimulatory polysaccharides, we tested the requirement for TLR and Dectin-1 pathways for these responses. First, we characterized the dependence upon the TLR signaling pathways. We found that mice deficient in TLR4, but not in TLR2, and, to a lesser degree, MyD88 or TRIF, showed a substantial decrease in myeloid

cell recruitment. Similar to previous reports on plant polysaccharides with TLR4 activity, we found that at least some serum proteins were required for this response.[277] Furthermore, we found that removal of acetate groups attenuated the cytokine response from this polysaccharide.

We next sought to determine if Dectin-1 could be involved in the response to AcaiPS. We identified the presence of β-glucan linkages in AcaiPS that were able to bind the Dectin-1 binding pocket, leading to ROS and syk induction by AcaiPS. To differentiate these responses from the TLR4-binding response, we measured these responses under serum-free conditions. In the absence of serum, AcaiPS, but not LPS, activated ROS and the syk pathway. These results indicate that AcaiPS mediates its effects through cooperative signaling of TLR4 and the phagocytic receptor Dectin-1.

## Methods

### Preparation of Agonists

Particulate zymosan (Sigma Aldrich, St. Louis MO) was prepared fresh before all experiments by suspension in DPBS. Ultrapure LPS from *E. coli* 0111:B4 (uLPS; Invivogen, San Diego CA) is devoid of other TLR agonists, and was used in these studies. Laminarin (Sigma) was dissolved in DPBS and stored at -80 for up to one month or used immediately.

AcaiPS was prepared as previously described.[9] Briefly, crude polysaccharide was prepared from dried acai fruit (acaiberrypure.com) by water extraction and triple precipitation in 70% EtOH. This crude polysaccharide was then purified by adsorption to

DEAE cellulose and elution with NaCl. The resulting polysaccharide was dialyzed against three exchanges of DPBS using a 6-8kDa MWCO membrane (Spectrum Labs, Rancho Dominguez, CA). The resulting Acai polysaccharides (AcaiPS) were dried by rotary vacuum concentrator (Savant/Thermo, Waltham MA) and stored at -20 until reconstitution in DPBS. AcaiPS was confirmed, as previously described,[9] to contaim less than 0.001EU LPS/µg AcaiPS by Pyrochome LAL with Glucashield (Both from Associates of Cape Cod, East Falmouth, MA). Reconstituted AcaiPS was stored at 4°C for up to 6 months with no apparent loss in activity.

Lung Inflammation Assay

Mice deficient in TLR4 (B6.B10ScN-Tlr4$^{lps\text{-}del}$/JthJ, Jackson), TLR2(B6.129-Tlr2$^{tm1Kir}$/J, Jackson), MyD88 (provided by Dr. Kieren A. Marr),[410] TRIF(Ticam1$^{Lps2}$/J, Jackson) or the background strain, C57BL/6, as well as HeJ (Charles River) and HeOuJ (Charles River) mice, were instilled intratracheally (i.t.) with 500µg Acai-PS, Acai-NaOH, or other polysaccharides 24h prior to collection of bronchoalveolar lavage fluid (BALF) and lung tissue. Prior to tissue collection, mice were euthanized by $CO_2$ asphyxiation. BALF was collected by lavage with two 1mL washes of HBSS containing 2mM EDTA. 1.7 to 2.0mL lavage fluid was recovered from each wash. BALF was centrifuged and the supernatant fluid was saved for IL-12 ELISA (IL-12(total); C15.6/C17.8-biotin, MabTech; Nacka Strand, Sweden). Next, lung tissue was collected by mincing with scissors then digestion for 1h in collagenase/DNase medium [200U/mL collagenase (Worthington Biochemical; Lakewood, New Jersey) and 0.08U/mL DNase

(Promega; Madison, Wisconsin) in RPMI with 20mM HEPES] at 37$^{o}$C. The resulting product was then passed through 35µm Nitex® nylon mesh (Sefar America; Depew, NY) to remove tissue debris. The pelleted BALF and lung cells were treated for 10m with cold ACK buffer to lyse red blood cells prior to analysis by flow cytometry. To analyze the cellular composition of the tissue and BALF, cells were stained with CD11b-FITC (M1/70; Becton Dickinson) and CD11c-APC (HL3; Becton Dickinson) prior to data collection using a FACSCalibur cytometer and analysis using CellquestPro software (both from Becton Dickinson).

β-glucan Inhibition ELISA

β-(1-3)glucan was measured using a capture ELISA similar to that described by Meikle *et al.*[411] To this end, 96-well MaxiSorp plates (Nunc, Thermo Scientific) were coated overnight with laminarin (Sigma) freshly reconstituted to 2µg/mL in PBS. Plates were then blocked for 90m with 1% BSA in PBS. Next, 50µL polysaccharides or PBS were added, followed immediately by the addition of 50µL of 1µg/mL anti-β(1-3)-glucan (Biosupplies; Melbourne, Australia). After 90m, plates were washed twice, goat anti-mouse-HRP (Southern Biotech, Birmingham, AL) was added for 60m, and the plates were washed thrice. HRP was visualized by ABTS (Moss Inc., Pasadena, MD) in a microplate reader (SpectraMax Plus384; Molecular Devices, Sunnyvale, CA).

Cell Cultures

All cells were cultured at 37$^{o}$C in a humidified atmosphere containing 5% $CO_2$. Human monocyte-macrophage MonoMac-6 cells (DSMZ; Brunswick, Germany) were grown in complete RPMI (cRPMI; RPMI 1640 (Mediatech Inc.; Herndon, VA) supplemented with 10% (v/v) FBS, 10µg/mL bovine insulin, 100µg/mL streptomycin, and 100U/mL penicillin) containing 10µg/mL insulin. For primary cells, whole blood was collected from healthy human adult donors with ACT tubes (Becton Dickinson). Peripheral blood mononuclear cells (PBMCs) were separated from whole blood using Histopaque 1077 (Sigma-Aldrich) as per the manufacturer's instructions. Bone marrow cultures were collected from C57BL/6, TLR4$^{-/-}$, TLR2$^{-/-}$, and MyD88$^{-/-}$ mice.

Measurement of Dectin-1 Surface
Receptor Binding by Flow Cytometry

Freshly collected human PBMCs were either fixed (2% PFA) prior to or immediately treated with Acai, laminarin, zymosan, curdlan, or pullulan polysaccharides for 1-2h in 100µL PBS. After this incubation period, 100µL Dectin-1 mAb directed to the polysaccharide-binding pocket of the receptor (Clone 259931, R&D Systems) or mAbs to CD14, MHCII, CD11b, or PBS were added and incubated for an additional 10 minutes. Cells were washed with cold PBS containing 1% horse serum and analyzed for mAb binding by flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ).

Measurement of ROS and Cell Activation by Flow Cytometry

Flow cytometry was used to analyze the activation status of cell subsets in human PBMC cultures as previously described.[1,2] Briefly, cultured cells were stained with anti-γδTCR monoclonal Ab (5A6.E9; ATCC)[378] anti-CD3(UCHT1, Biolegend )and CD69 (FN50; Biolegend, San Diego, CA). Activation was measured by flow cytometry, using a FACSCalibur, as the increase of CD69 expression.

To analyze ROS production, Human PBMCs were cultured at $2x10^6$ cells per mL in serum-free XVIVO medium with various agonists or vehicle controls. Sixty minutes into the culture, 10μM aminophenyl fluorescein (APF; Invitrogen) was added and the cultures maintained for an additional 30m. Cells were then lifted with EDTA/PBS and kept on ice before being read by flow cytometry (FACSCalibur). Monocytes were selected by FSC/SSC gating. The percentage of monocytes producing ROS were determined by subtracting the percentage of untreated controls from the treated samples.

Determination of Acai-Induced Cytokine Production

Human PBMCs or MonoMac-6 human monocytic cells were plated in 96-well plates at a density $2\times10^5$ cells in 100μL per well. Cells were cultured for 24h and then supernatant fluids collected and analyzed for cytokines. Cytokine that were assayed included mouse TNFα(Biolegend), mouse IL-12 (Mabtech, p40: C15.6/C17.8), human TNFα(Biolegend) or human IL-12(Biolegend). To determine the requirement for receptors, hIgA anti-human blocking antibodies for TLR2, TLR4, and CD14, or non-specific antibody controls (all from Invivogen) were used in some experiments. In

experiments measuring the requirement for serum in cytokine responses, both bovine serum (BCS, Atlas) or human serum (Fisher) were used. For serum fractionation studies, 500µL of human serum was applied to a 2mL, 0.3cm diameter Sephadex 300HR column. Twelve fractions eluted with DPBS and collected over 90 minutes at a flow rate of 50µl/min. Protein concentration was measured by BCA.

Measurement of Phosphorylated- and Total- Syk

Monocyte-enriched cells were isolated from PBMCs by adherence to tissue culture flasks. To this end, freshly collected PBMCs were cultured in tissue culture flasks with cRPMI for 30m, and the supernatant fluid and non-adhered cells removed. Adherent cells were then collected after 2mM EDTA wash. Cells were 30% to 50% positive for Dectin-1, as determined by antibody staining and analysis by flow cytometry. Cells were then resuspended in 2mM sodium orthovanadate (Acros Organics, Geel Belgium) to inhibit phosphatases and cultured for 60m with agonists ($5x10^6$ cells/mL, $1x10^7$ total). After culture, cells were pelleted by centrifugation, and lysed on ice with 100µL CERI/II lysis buffers containing HALT protease and phosphatase inhibitors (all from Pierce). The supernatant fluids were saved at -80C until analysis. To analyze phospho-syk or total-syk content, samples were denatured in 10x denaturing buffer (Becton Dickinson) at 90°C for 5m. Twenty µL of each sample, or standard curves were then stained with beads for phospho- or total-syk (Becton Dickinson) as per the manufacturer's protocol. The beads were then analyzed using a FACSCalibur flow cytometer (Becton Dickinson) and content determined using the standards. In assays measuring both phospho- and total syk, syk

activation is represented as the percent of syk phosphorylation (phospho-syk/total-syk*100%). In the experiment measuring only phosphorylated syk, data are represented as the international units (IU) of syk expressed per $1x10^6$ lysed cells.

## Results

### AcaiPS-Mediated Lung Activation is Diminished in TLR4-Deficient Mice

To determine if AcaiPS activation of the mouse lung was dependent upon TLR4, we tested mice deficient in aspects of the TLR4 signaling system. Previously, we identified an increased population of $CD11c^+$ myeloid cells in the lungs of AcaiPS-treated mice. To determine if this recruitment was altered in mice deficient in the TLR4 pathway, wildtype and various knockout mice were treated with AcaiPS or vehicle for 24h prior to the collection of lung BAL. Compared to wildtype, mice functionally deficient in TLR4 (HeJ) showed a diminished capacity to recruit $CD11c^+$ cells to the lung after AcaiPS treatment (Figure 6.1A). Mice deficient in either of the downstream mediators, TRIF or MyD88 did present with a diminished response compared to the $TLR4^{-/-}$ mice, however these were not as striking (Figure 6.1B). We also previously demonstrated an increase in IL-12 production from mice treated with AcaiPS. This cytokine response was likewise attenuated in TLR4-deficient mice compared to wildtype (Figure 6.1C).

To confirm the dependence of these responses on TLR4, we next measured the production of the TLR4/NFκB-associated cytokine TNFα in peritoneal cells. In these experiments, we included LPS and zymosan to function as controls for Dectin-1- and

TLR4-mediated signaling pathways. Peritoneal cells from TLR2- and MyD88-deficient mice treated with AcaiPS produced TNFα similar to wildtype, however TLR4$^{-/-}$ mice showed an attenuated cytokine response (Figure 6.2). These results indicated that mouse populations from two different tissues could activate TNFα production during AcaiPS culture via a TLR4-dependent route.

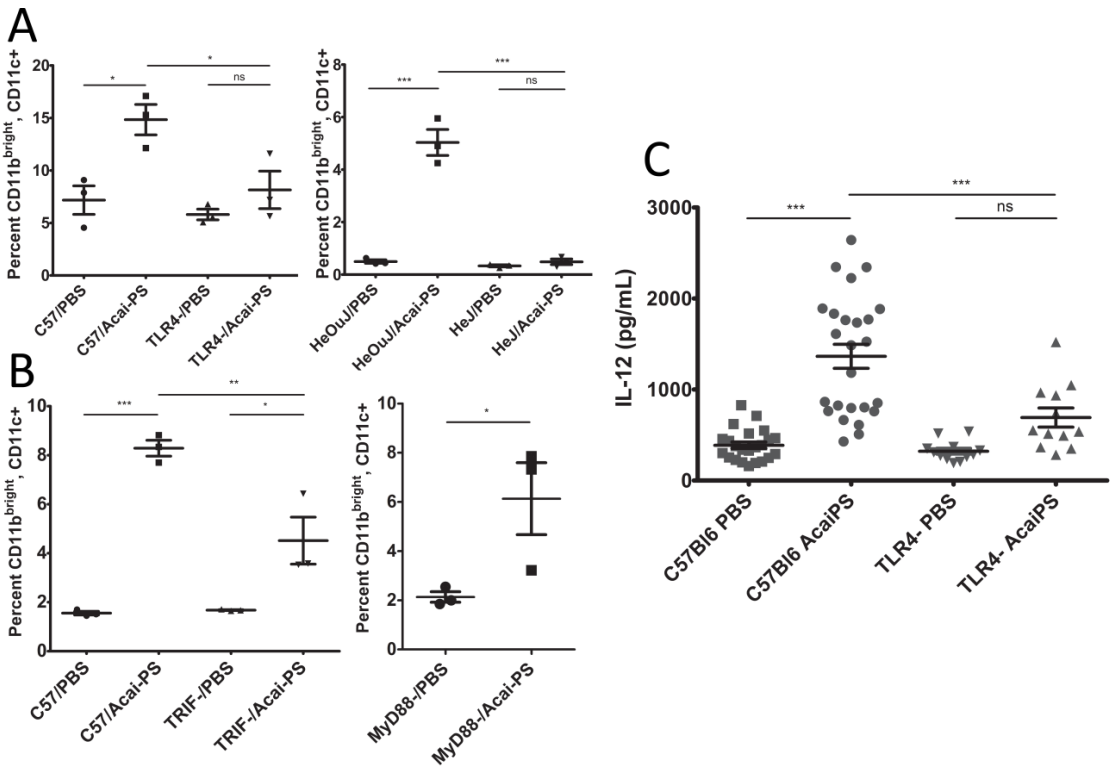
## Human Cells are Likewise Dependent upon TLR4 for AcaiPS Cytokine Responses and Require Polysaccharide Modification for Activity

Ligand recognition by TLR4 can vary between humans and mice,[412,413] therefore, we next tested these cells in the human macrophage-like cell line, MonoMac-6 (MM6), to confirm relevance to human responses. First, we sought to determine if, similar to other reports with plant polysaccharides, AcaiPS was dependent upon serum proteins for optimal responses. Comparable to ultrapure LPS (uLPS), the production of TNFα by MM6 cells was diminished in the absence of LPS-binding proteins (Figure 6.3A). The dependence upon TLR4 and its accessory protein, CD14, was measured using blocking antibodies. A decrease in TNFα production was observed when antibodies against the LPS receptors, but not when anti-TLR2 or a non-specific antibody were added to the culture (Figure 6.3B). Although MM6 cells express CD14 on the surface, the diminished response in serum-free cultures indicated an additional LPS co-receptor might be involved. This could include MD-2 and/or LBP. Therefore, human serum was fractionated by Sephadex 300 and the fractions added to otherwise serum-free culture

conditions. Increased TNFα production in AcaiPS correlated similarly to that of LPS, indicating AcaiPS uses serum binding proteins similar to LPS.

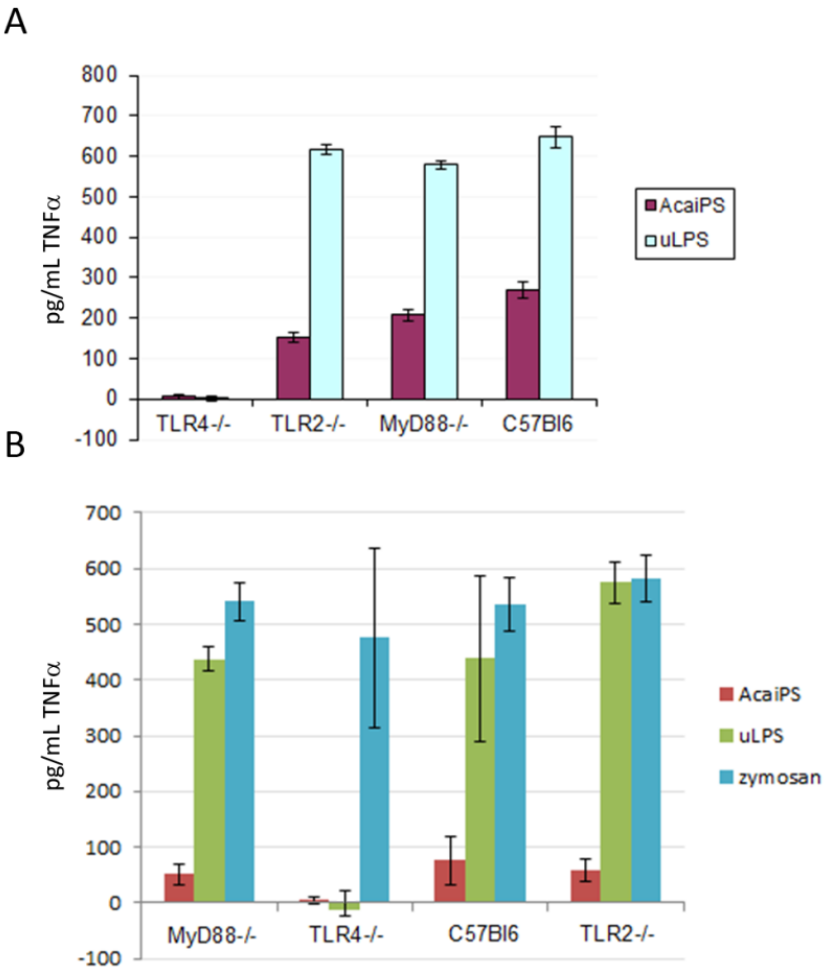
A non-LPS, *Klebsiella* capsular polysaccharide is able to activate TLR4 via unique acetate and/or pyruvate modifications of the polysaccharide. AcaiPS also contains acetate side chains.[9] To determine if a similar structure was responsible

Figure 6.1 AcaiPS Lung Activation is reduced in TLR4-Deficient Mice




Mice were treated with 500µg of AcaiPS for 24h prior to the collection of BAL and lung tissues. A and B) Cell populations from collagenase-digested lung tissue were analyzed by flow cytometry using antibodies to CD11b and CD11c. The percentage of $CD11b^{+}$, $CD11c^{+}$ cells are presented in each graph. A) TLR4-sufficient (C57BL/6, HeOuJ) and TLR4-deficient ($TLR4^{-/-}$, HeJ) populations in AcaiPS and PBS-treated mice. B) Mice deficient in the TLR4 downstream adaptors, TRIF or MyD88. C) BAL from TLR4-sufficient (C57BL/6) and TLR4-deficient ($TLR4^{-/-}$) mice treated with AcaiPS or PBS-were tested for IL-12 production. P-values were determined by ANOVA/Tukey's or Student's T test as appropriate.

Figure 6.2 AcaiPS Activates Peritoneal Cells to Produce TNFα in a TLR4-Dependent Manner



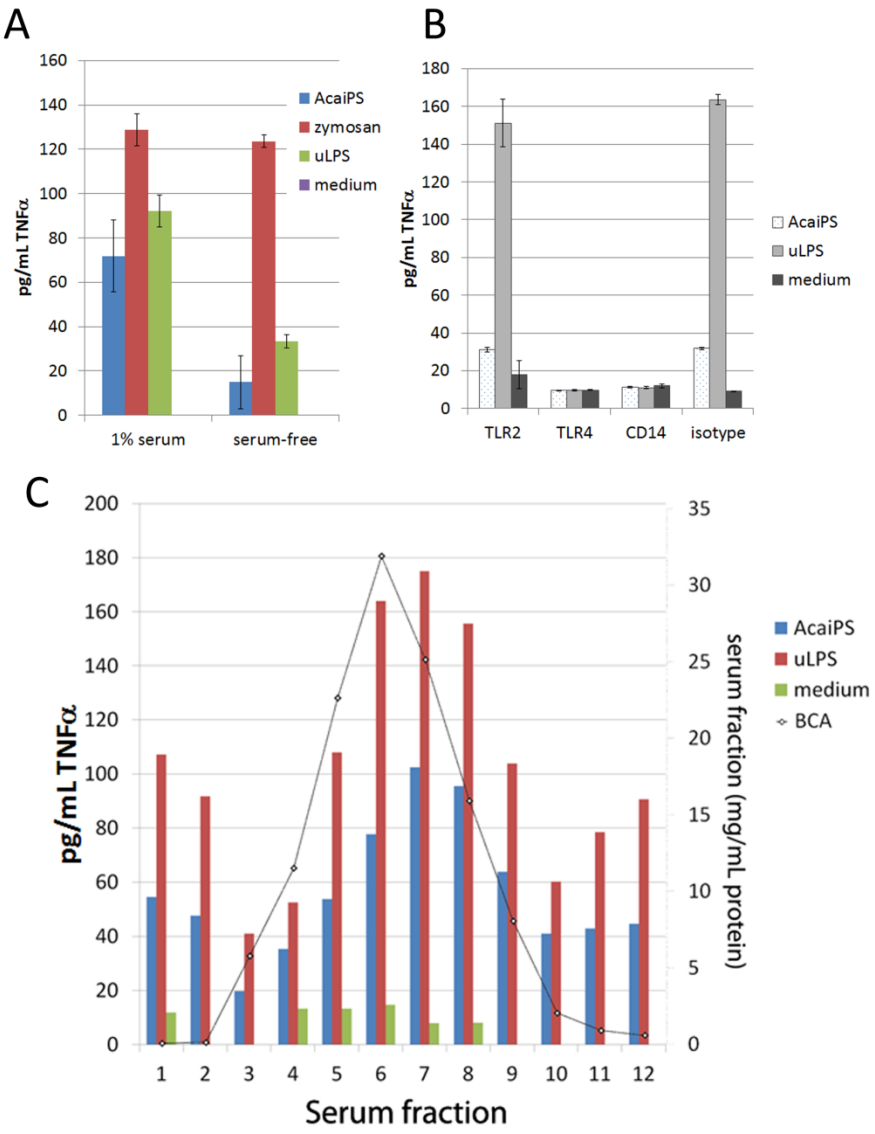

Peritoneal cells were collected from naive mice and cultured at $1x10^6$ cells/mL for 24h with the identified agonists: AcaiPS 5µg/mL, uLPS 1ng/mL, zymosan 5µg/mL, or medium only. Peritoneal cells from three animals belonging to each strain were collected. Data represent the average cytokine production from the agonist-treated cultures minus the donor-specific medium control. A) TNFα cytokine ELISA data from cultures assaying the response to AcaiPS or uLPS. B) Repeat of experiment in A except that zymosan was included as an agonist. Data represent the average response for each strain, error bars represent the SEM.

for AcaiPS activity, we treated the polysaccharide with NaOH to cleave acetate residues. AcaiPS or AcaiPS treated with 1.0N NaOH (Acai-NaOH) were cultured with human PBMCs and activation of the γδ T cell population was measured as previously described.[9] Cells treated with AcaiPS, but not Acai-NaOH increased expression of the activation marker, CD69 (Figure 6.4A). NaOH can disrupt secondary structure of polysaccharides at high concentration (>0.1N). Therefore to determine if this response was dependent upon secondary structure, we next prepared buffer-, 0.1N NaOH-, and 0.01N NaOH-treated AcaiPS, as well as similarly-treated uLPS and zymosan, which served as controls. MM6 cells treated with AcaiPS-NaOH produced less TNFα than those cultured with buffer-treated AcaiPS. MM6 cells cultured with NaOH-treated uLPS or zymosan produced similar, albeit slightly less TNFα than HEPES-treated agonists (Figure 6.4B). Removal of acetyl groups was confirmed by alkaline hydroxylamine assay (Figure 6.4C).[414]

## Phagocytic Responses to AcaiPS are Independent of Acetylation and Serum Proteins

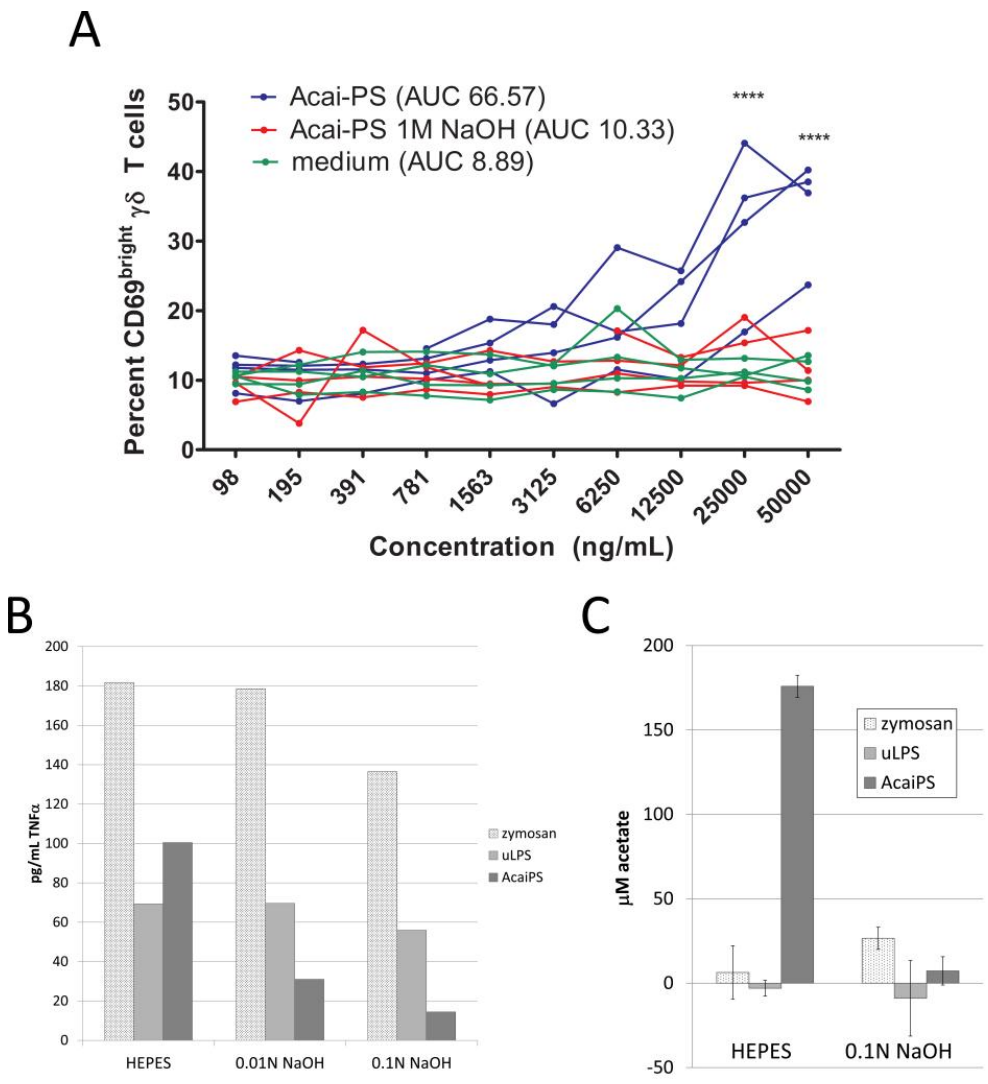
In addition to cytokine responses, TLR4 can activate phagocytic responses involving syk and ROS production. To determine the activation status of syk, human PBMCs, enriched for monocytes, were cultured in serum-containing medium with zymosan, AcaiPS, AcaiPS-NaOH, LPS or the negative controls laminarin or PBS. After 60m, lysates were collected, and syk/phospho-syk were analyzed. In zymosan, LPS, and AcaiPS-treated cells, syk was phosphorylated when cultured in the presence of serum (Figure 6.5A). Unexpectedly, AcaiPS-NaOH also caused the phosphorylation of syk.

Figure 6.3 AcaiPS Recognition Utilizes TLR4 and Co-Receptors




MM6 cells were treated with AcaiPS, uLPS, zymosan, or medium only for 24h under conditions to measure the requirement for TLR4 co-receptors. TNFα was then measured from the supernatant fluids by ELISA. A) MM6 were cultured in the absence or presence of 1% serum with and the various agonists. B) MM6 were cultured in 1% serum with antibodies to the various PRR or isotype control during culture. C) Human serum was fractionated by Sephadex 300, analyzed for protein content by BCA (black line), and added to otherwise serum-free cultures containing medium only, AcaiPS or uLPS.

Figure 6.4 Deacetylation of AcaiPS by NaOH Attenuates Activity.




Acai-PS was treated with 0.01, 0.1, or 1.0M NaOH to remove acetate groups and tested for activity. A) The degraded (1.0N NaOH) and non-degraded AcaiPS preparations were then titrated in 48h PBMC cultures experiments to measure γδ T cell activation (CD69 expression). Results are pooled from two experiments with two donors per experiment. B) AcaiPS, uLPS, or zymosan were treated under neutral (HEPES buffer) or basic conditions (0.1N or 0.01N NaOH) for 2h. The resulting preparations were then added to cultures of MM6 cells, cultured for 24h, and then supernatant fluids were analyzed for TNFα by ELISA. n=1. C) Preparations used in B were assayed for acetylation. AcaiPS-HEPES was more acetylated than all other samples ($p>0.001$) by ANOVA/Tukey's.

This was contrary to the previous results showing TNFα and γδ T cell activation was attenuated in these preparations.

Syk activation and ROS generation by LPS are dependent upon CD14 and, in the case of ROS, LBP.[61,62] To determine the role of serum in these phagocytic responses to AcaiPS, cultures were prepared in serum-free medium and then analyzed for phospho-syk. In these cultures, zymosan and AcaiPS cultures contained increased phospho-syk, whereas LPS-treated cultures did not (Figure 6.5B). This suggested the activation of syk followed a different pathway than is typical for TLR4. Next, ROS production was analyzed in human PBMCs treated in the absence of serum. Zymosan- and AcaiPS-treated monocytes produced ROS, as measured by Aminophenyl fluorescein (APF) staining, however LPS-treated monocytes did not (Figure 6.5C and D). These results indicated that AcaiPS activates the phagocytic pathway independently of NaOH degradation and TLR4 accessory proteins.

Since AcaiPS retained the ability to induce phagocytic activity and ROS production in the absence of serum proteins and acetate degradation, we next sought to determine if AcaiPS could bind to an alternate receptor. To this end, we tested Dectin-1 binding by AcaiPS. First, we tested whether AcaiPS contained the appropriate linkages for recognition of Dectin-1 using an inhibition ELISA with a mAb directed against β(1-3)glucan that does not cross-react with β(1,3-1,4) glucan linkages.[411] AcaiPS, as well as the polysaccharides zymosan [β(1,3-1,6)], pullulan [α(1,4-1,6)], and laminarin [β(1,3-1,6)], were assayed for the ability to block binding to laminarin-coated plates. Pullulan, which does not contain β-glucan linkages, served as a negative control, and did not

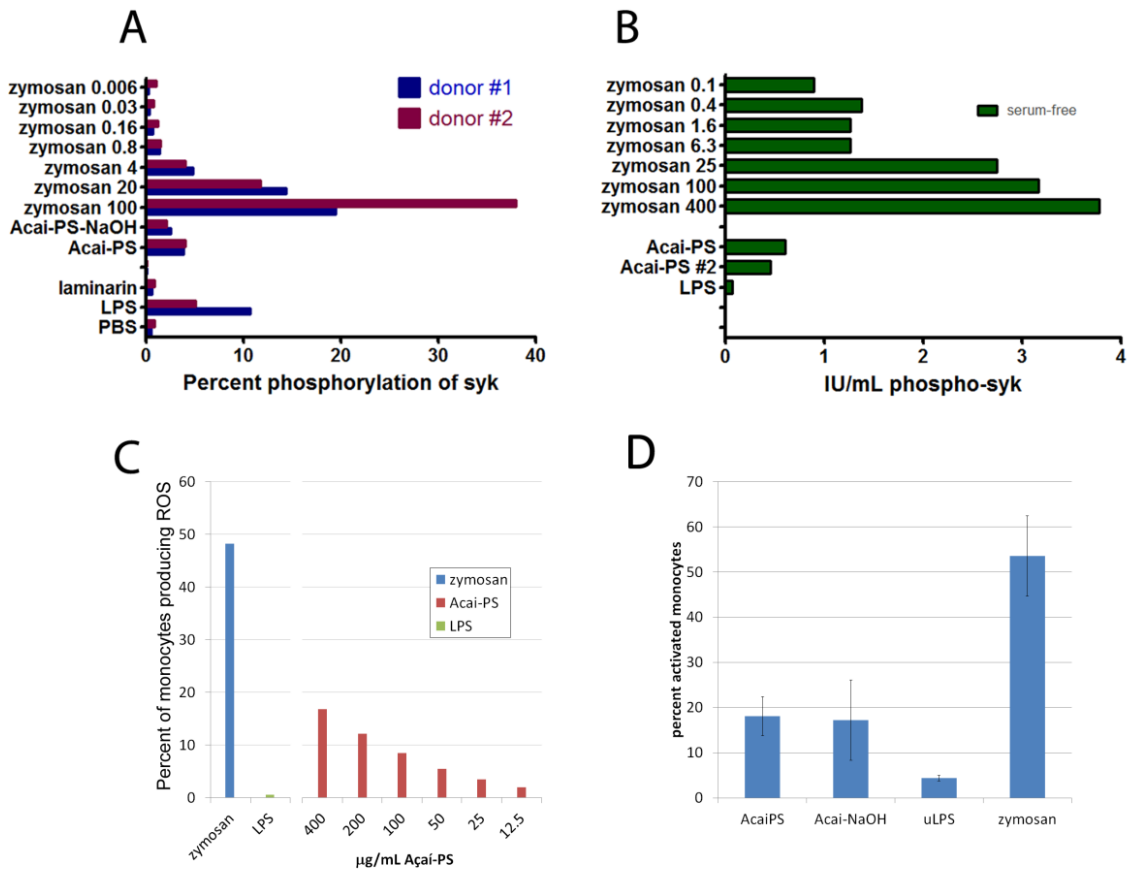
prevent binding. Alternatively, AcaiPS, zymosan, and laminarin all decreased antibody binding, indicating the presence of β-glucan residues (Figure 6.6A). To determine if AcaiPS could also bind the Dectin-1 receptor, human PBMCs were cultured with the same polysaccharides and the binding affinity of an antibody to the Dectin-1 binding domain, as well as other myeloid cell receptors, were analyzed by flow cytometry. Laminarin, zymosan, and Acai-PS were able to sterically inhibit binding of the Dectin-1 antibody, but not the binding of other monocyte receptors (Figure 6.6B). Inhibition of Dectin-1 binding was confirmed in replicate experiments (Figure 6.6C).

To determine the relative role for syk in the production of TNFα during AcaiPS culture, human PBMCs were cultured with AcaiPS, or separate agonists for Dectin-1 (zymosan) or TLR4 (*E. coli* LPS). Analysis of TNFα production showed that syk decreased the TNFα production of all of these agonists, however, this was most prominent with AcaiPS in human PBMCs (Figure 6.7).

## Discussion

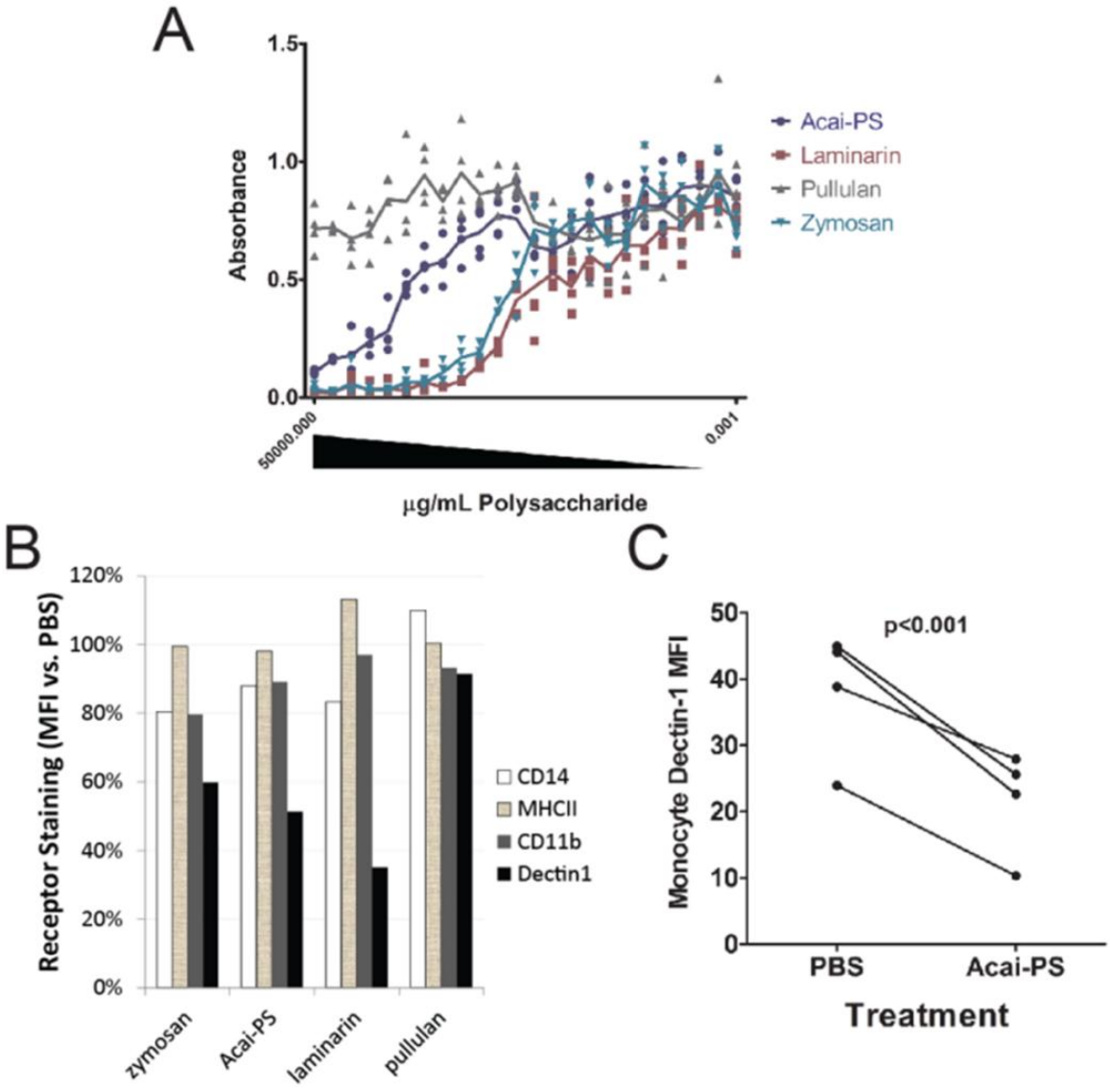
The recognition of ligand by PRR is required for appropriate responses to a variety of pathogens. This recognition event can lead to varied responses including endocytosis, antiviral cytokines, antibacterial cytokines, and, in most instances, promote the formation of the adaptive immune response. However, in some instances, ligand binding can lead to a minimal response, particularly at sensitive sites, such as the lung. These responses are controlled by suppressive factors and the cooperative activities of the PRR. Characterizing the receptors tuned to each innate agonist can ultimately improve our

Figure 6.5 AcaiPS Activates Syk and Generates ROS in the Absence of Serum Proteins




A) Monocytes were enriched from human PBMCs by adherence to tissue culture plastic. Cells were then treated with orthovanadate and agonists (uLPS 100ng/mL, zymosan: 6ng-100µg/mL, all other polysaccharides: 100µg/mL) for 60m in serum-containing medium. Cell lysates and standards were analyzed for total- or phospho-syk by bead array. Data represent the percent syk phosphorylation (phospho/total). B) Lysates and standards were prepared as in A, except in serum-free conditions and measuring only phosphorylated syk. n=1. C and D) Activation of ROS in monocytes from serum-free human PBMC cultures were measured by APF staining. Monocytes were gated by FSC/SSC and ROS measured by fluorescence C) Cells were treated with 50µg/mL zymosan, 1µg/mL uLPS, or titrations of AcaiPS (12.5 to 400µg/mL). Data represent the percent of ROS-producing monocytes minus background activation (vehicle control) D) Repeat of C in three separate experiments from different donors measuring 500µg/mL AcaiPS or AcaiPS-NaOH, 1µg/mL LPS, or 50µg/mL zymosan. Error bars represent SEM.

Figure 6.6 AcaiPS Contains β-glucan Structures and Binds to the Dectin-1 Receptor.



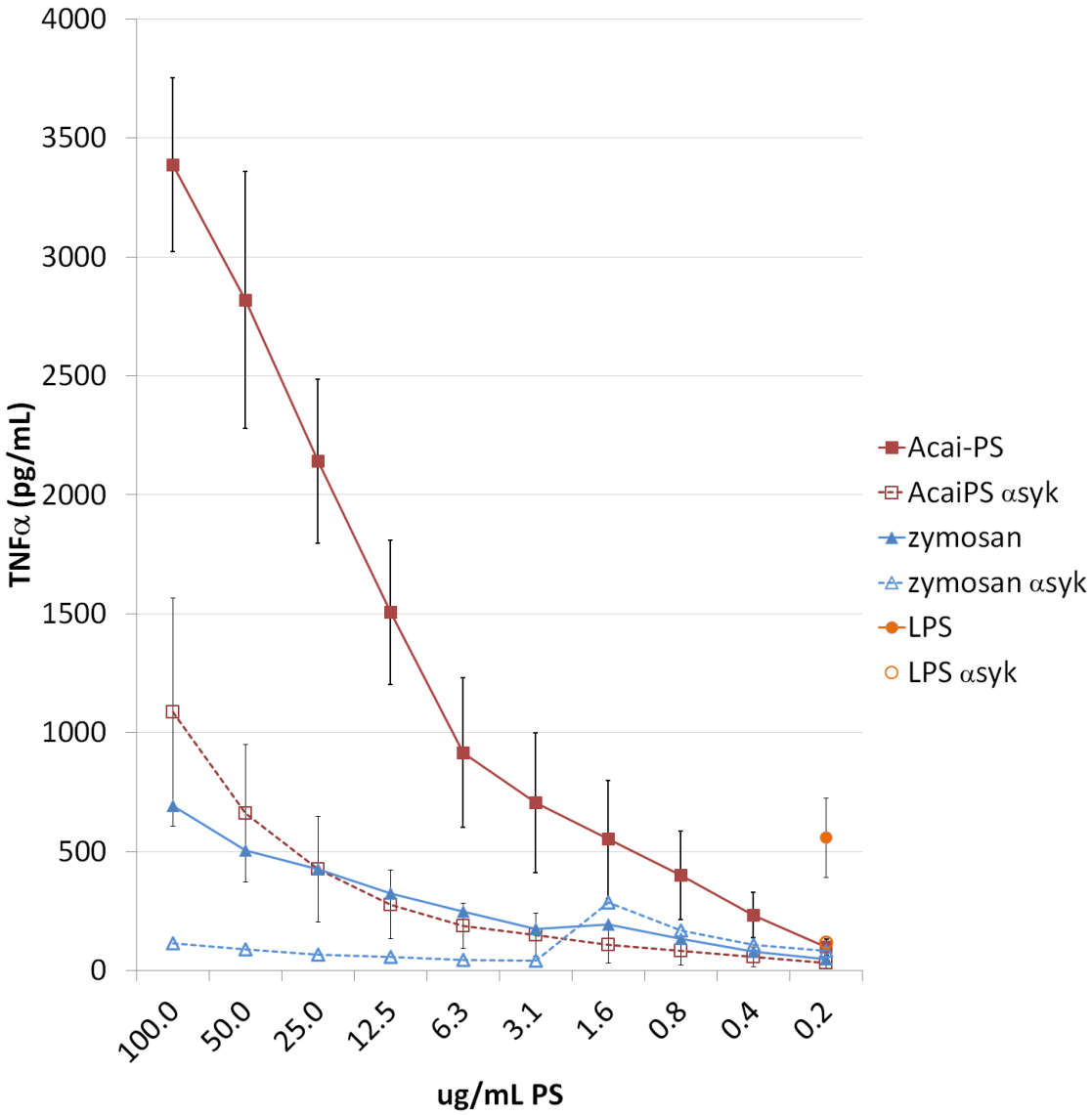

A) β(1,3)-glucan content was measured by inhibition ELISA. Laminarin-coated plates were co-incubated with anti-β(1,3)glucan mAb and dilutions of polysaccharides. mAb binding to the plates was measured by anti-mouse-HRP and TMB. Results are representative of three similar experiments. B,C) Human PBMCs were treated with polysaccharides or PBS for 1h and then stained with anti-Dectin-1 or other antibodies prior to analysis of monocytes by FACS. B) mAb staining (as a function of PBS) for four surface markers and four polysaccharides. C) Dectin-1 mAb staining in PBS or Acai-PS-treated cells.

understanding of innate immune responses.

Polysaccharides from multiple species including bacteria, fungi, and plants have immunomodulatory activity. Here we elaborate upon the polysaccharide fraction obtained from Acai. The Acai polysaccharide preparation is water soluble and composed primarily of arabinose, galacturonic acid and galactose saccharides with trivial amounts of endotoxin, polyphenols, or protein. Previously, we identified innate stimulatory activity in this polysaccharide fraction, including γδ T cell and monocyte activation. Acai-PS induces IL-6, TNFα, IL-10, and ROS production in human PBMC cultures. *In vivo*, Acai-PS induces myeloid cell recruitment and IL-12 production in the lung.

In this report, we show that AcaiPS activates mouse and human myeloid cells via TLR4 signaling. We also show that this response is dependent upon CD14 and other serum-soluble LPS-binding proteins. Others have shown that TLR4 can recognize multiple structures.[275,276] In addition to endotoxin, TLR4 can recognize hyaluronic acid, heat shock proteins, and other polysaccharide structures, including the capsular polysaccharide structure of *Klebsiella*. This polysaccharide antigen from *Klebsiella* consists of repeating trisaccharides with extensive acetylation and pyruvylation. Our previous report showed that AcaiPS contains similar saccharide subunits by $^{1}$H NMR, acetylation.[9] Treatment with NaOH removed AcaiPS acetylation, attenuated TNFα production in monocytes, and attenuated γδ T cell activation. This suggested that, similar to *Klebsiella* capsular polysaccharide, acetylation is required for recognition of the AcaiPS by TLR4.

Figure 6.7 AcaiPS-Induced TNFα Production is Partially Dependent upon Syk




Human PBMCs at $2x10^6$/mL were cultured with various concentrations of AcaiPS, zymosan, or *E. coli* LPS for 24h in serum-containing media. In addition to the agonists, cells received either vehicle only or 1μM syk inhibitor (αsyk). TNFα was measured from the cell supernatants by ELISA. Data represent the average concentration of TNFα from three donors, Error bars (not shown in zymosan cultures for the sake of clarity) represent the SEM.

However, when measuring endocytosis-associated responses in monocytes, NaOH treatment and serum depletion did not attenuate AcaiPS activity. These results prompted us to look for additional phagocytic receptors for AcaiPS. Since other reports show that soluble plant β-glucans can mediate Dectin-1/syk activation, we tested for similar activity from AcaiPS. These experiments identified β-(1,3) glucan linkages in AcaiPS and the ability of this PS to bind to the Dectin-1 receptor.

These exists a divergence between plant and fungal polysaccharide recognition by Dectin-1. Whereas fungal polysaccharides require particulate β-glucan for most Dectin-1 responses, including syk activation and NFκB production, numerous soluble plant polysaccharides are shown to activate robust cell responses through Dectin-1. In particular, Tada *et al*. show that barley β-glucan activates syk and NFκB in Dectin-1/syk/CARD9/Bcl10, but not syk/CARD9/Bcl10-transfected 293T cells. These differences in Dectin-1-mediated responses may be due to the engagement of different co-receptors or possibly due to endocytosis-independent signals.[79]

The observation that AcaiPS can modulate both TLR4 and Dectin-1-mediated signaling events may shed some light upon how these Dectin-1 ligands produce different responses. Whereas fungal polysaccharides do not utilize the TLR4 receptor, plant polysaccharides are described that utilize TLR4 as well as other co-receptors. Although the TLR4 activity of AcaiPS is absolved after de-acetylation by NaOH treatment, it remains unclear what components of the TLR4 complex no longer recognize the polysaccharide. It is possible that components of this pathway remain able to bind

AcaiPS and that these lead to the more robust response in Dectin-1 that is not observed by soluble fungal β-glucans.

The end result of a shared TLR4/Dectin-1 activation profile is intriguing. Many have shown accentuation of NFκB-associated signals via TLR/Dectin-1 co-signaling,[74,77] however, an additional level of cell response occurs during engagement of these multiple receptors. Of particular interest in these studies is the observation of Dectin-1 activation of the Raf-1 pathway, which increases TLR4-mediated IL-12p35 and IL-12p40 production and promotes Th1 maturation.[75] As shown here and previously,[9] AcaiPS induces IL-12 production in the lung, suggesting a similar response may occur *in vivo*.

In parallel to these studies, we worked with Skyberg *et al*.[415] to test the ability of AcaiPS to protect against infection by the intracellular pathogen, *Francisella tularensis*. *F. tularensis* clearance is highly dependent upon IL-12 and IFNγ production for DC maturation and systemic protection.[416-418] In fact, the addition of IL-12 alone can prolong survival during challenge.[419] In this study, we found that therapeutic and prophylactic treatment with AcaiPS protected mice against *F. tularensis* challenge.[415] This response was associated with the production of the Th1 cytokine, IFNγ, by lung NK and γδ T cells. Furthermore, protection could be reversed by antibody-mediated clearance of IFNγ. The generation of this robust IFNγ response by the TLR4/Dectin-1 agonist AcaiPS indicates it may serve as a novel approach for generating innate immune responses that are protective in disease.

Acknowledgements

MyD88$^{-/-}$ mice were kindly provided by Dr. Kieren A. Marr, Division of Infectious Diseases, Oregon Health and Science University, Portland, OR. We would like to thank Kathryn Holderness for critical review of the manuscript, as well as Dr. Rob Cramer and Kelly Shepardson for valuable comments on host mechanisms of fungal polysaccharide recognition.

# CHAPTER SEVEN

# THERAPIES BASED ON THE REGULATION OF INNATE IMMUNITY BY OLIGOMERIC PROCYANIDINS AND ACAI POLYSACCHARIDES

## Abstract

The *in vitro* activation of the innate immune system by plant OPCs and polysaccharides are markedly different. In OPCs, this response leads to a priming effect in γδ T cells typified by an increased stability of inflammatory genes. With polysaccharides, a TLR4- and Dectin-1-mediated response is developed that leads to generation of Th1-associated cytokines in the mouse lung. How these immune responses can be therapeutically applied to disease models has been addressed by collaborators. Jodi Hedges has demonstrated that OPCs can not only prevent dengue virus entry into human PBMCs, but has also indicated an increased innate antiviral response in OPC-treated cells[249]. Emily Kimmel has followed up on the observation of high GM-CSF induction upon OPC treatment to develop a potential therapy for low neutrophil numbers (neutropenia) associated with malnutrition and chemotherapy. Her studies indicate that OPCs can rapidly reconstruct the neutrophil populations of chemically-depleted mice (unpublished data). Finally, Jerod Skyberg *et al*.[240] show the therapeutic potential for both OPCs and polysaccharides in disease models for IBD and *Francisella* lung infection, respectively.[415] These collaborative studies indicate that the

immunostimulatory effects of OPCs and polysaccharides can be successfully applied to treat *in vivo* models of disease.

## Therapeutic Treatment of Neutropenia with OPCs

Neutropenia is a worldwide health problem. The neutrophil is the most potent effector cell of the immune system and is required for optimal early responses to infection. Leading causes of neutropenia, or low neutrophil counts, are poor nutrition and chemotherapy. Current treatment for neutropenia consists of recombinant CSF, which causes the release of neutrophils from the bone marrow. GM-CSF has similar activity, and is effective in treating febrile neutropenia.[420] Unfortunately, due to the expense of recombinant proteins, treating malnutrition with these proteins is unattainable. Therefore, a cost-effective therapy would be of benefit for this disease. Since OPC sources are widely available and are able to induce *GMCSF* production, we sought to characterize the potential of APP to induce a similar release of neutrophils from the bone marrow.

Since GM-CSF is able to induce neutrophil expansion and increased *GMCSF* transcript production and stabilization were previously shown to occur *in vitro* as a result of OPC culture, we sought to determine if GM-CSF production occurs *in vivo*. To this end, APP was injected into the peritoneum of $CXCR2^{-/-}$ mice (Jackson Lab via Allen Harmsen). These mice were used to prevent neutrophil recruitment[250] and thus cellular contamination of the resident population. In these studies, we found an increase in *GMCSF* transcript significantly greater in APP-treated animals than TNFα-treated or

control animals (Figure 7.1A). We did not detect significant amounts of GM-CSF protein in the peritoneum of APP-treated mice or in *ex vivo* cultures with peritoneal or bone-marrow-derived cells, suggesting secondary events were necessary for *GMCSF* translation (data not shown).

To determine if APP-generated *GMCSF* transcripts could have an impact during disease, these OPCs were used to treat a mouse model of neutropenia. Disease was induced via antibody-mediated neutrophil depletion. Concurrently, mice were treated with APP or vehicle. After 45h, the mice receiving APP did not demonstrate the reduced neutrophil counts associated with the vehicle-treated mice (Figure 7.1B, Emily Kimmel). These results suggest a potential therapeutic effect from APP-derived *GMCSF* transcripts or transcript stabilization. These unpublished data are preliminary in nature, but suggest a possible therapeutic application for the abundant increase in *GMCSF* transcripts associated with OPC responses.

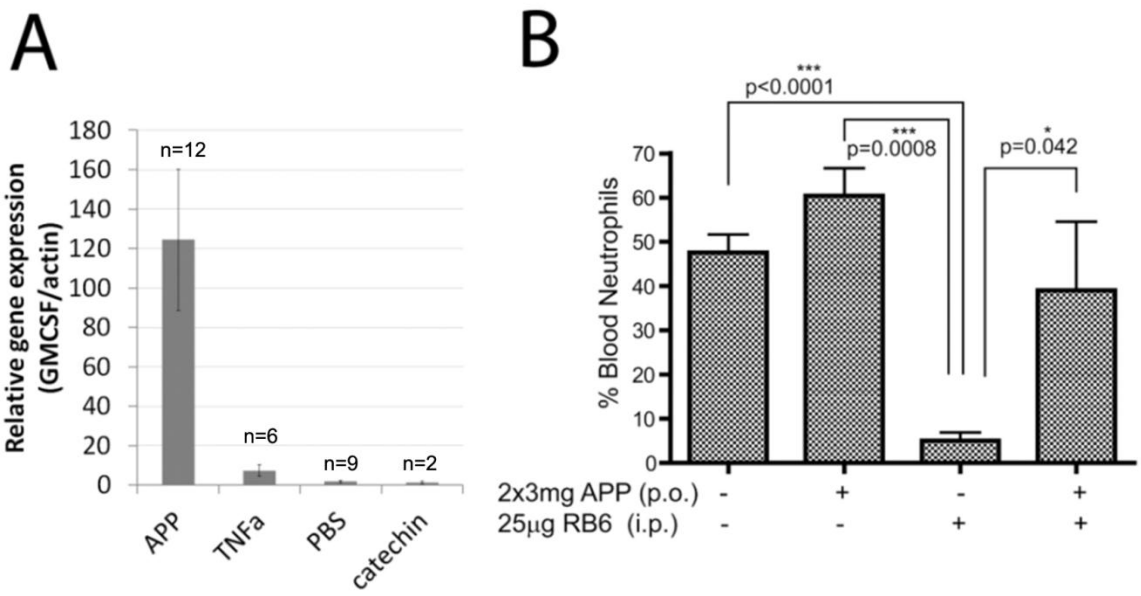
## Antiviral Responses to OPCs

OPCs can directly interact with numerous viral capsids and prevent viral infection. To determine if the immunostimulatory effects of OPCs could play an additional role in preventing viral infection, Kimmel *et al.*, tested the response of human PBMCs to dengue virus in the presence of OPCs under different conditions.[249]

Their first observation was that OPCs could directly inhibit viral entry. This was not unexpected, and was in agreement with previous reports demonstrating that they bind to and prevent viral penetration into cells.[421,422] Next, they treated human PBMCs with

OPCs, removed the OPCs, and then measured viral infection (unpublished data). Cells pre-treated with OPCs, but not those treated with vehicle, were more resistant to viral infection.

Figure 7.1 APP-Mediated Neutrophil Recovery during Neutropenia.




A) CXCR2-/- mice were injected i.p. with 500µg APP, 500ng TNFα, H2O or 500µg catechin. After 4h, mice were sacrificed and peritoneal cells were collected by cavity wash. RNA was collected and analyzed by qPCR for GMCSF relative to beta actin. Data are cumulative from two independent experiments with an n of at least 3 per experiment except catechin, which was tested in only 1 experiment. * $p<0.05$ by 1-way ANOVA. B) The Ly6G+ cells of 5 week old female BALB/c mice (n=5) were depleted by injection with the mAb RB6-8C5. Concurrent with the injection, mice were given 3mg APP by oral gavage. A second 3mg dose was given 21h later. Plasma samples were collected 45h post mAb treatment, stained with antibodies specific for CD11b, CD11c, Ly6G and F4/80 and analyzed by flow cytometry.

They next measured the ability of OPCs to stimulate an innate antiviral response in infected human PBMCs. Under low multiplicity-of-infection (MOI) conditions, which

are comparable to early infection, they observed an increased expression of MHC Class I, TNFα, and *STAT1* in OPC-treated cultures. They also looked at the potential effect of OPCs at high MOI infection to simulate dengue hemorrhagic fever. In these experiments, they identified an increase in *IFNB* and *MX1* transcript expression. Combined, these results suggested OPC could accentuate innate immune responses to viral infection under high- and low-titer disease.

To determine the role of OPCs in a more general model of viral infection, they next tested whether the response to type I interferon was modified by OPC treatment. To this end, human PBMCs were treated overnight with either vehicle or APP, then briefly with Type-I IFN. In PBMCs treated with APP before Type-I IFN, a more than five-fold increase in the phosphorylation of the IFN-response element, STAT2 was observed compared to IFN alone. Direct OPC treatment likewise led to an increase in MHCI expression in IFN-treated or -untreated cultures. These results show that OPCs promote a general antiviral effect that may have implications for numerous viral infections. Jodi Hedges is currently working to expand upon these studies using other models of viral infection and characterizing the mechanisms for OPC-mediated viral protection.

## Therapeutic Role of OPCs in Chemically-Induced IBD

Jerod Skyberg *et al.*[240] have looked into the potential therapeutic application of OPCs in the treatment of IBD. This work focuses on the DSS-induced colitis model. Using this model, Dr. Skyberg showed that, compared to untreated controls, OPCs could reduce weight loss and colonic inflammation caused by DSS treatment. They observed

decreases in transcripts for inflammatory cytokines (IFNγ, IL-17, TNFα and more) and chemokines (CXCLs 3 and 9-11) during OPC therapy. Interestingly, this response was observed only in mice receiving OPCs orally (*ad libitum* in drinking water or *per os*). When given OPC by peritoneal injection, mice did not show significant improvement in clinical score, suggesting the oral route was important for these effects.

To determine the cell population of the intestinal mucosa responsible for the protective effects of OPCs in DSS-induced colitis, the authors repeated the previous experiments in various knockout mice. The first series of these experiments showed that APP was protective in mice deficient in IL-10, γδ T cells, and IRF1 (required for appropriate development of cytotoxic T cells and NK cells) but the protective effect was diminished in mice deficient in both αβ and γδ T cells as well as B cells ($Rag1^{-/-}$ mice). The protective effect of OPCs was then shown to be lost in αβ T cell-deficient mice and that either CD4 or CD8 could contribute to the response to OPCs.[240]

In another series of experiments, the authors measured the effect of APP on inflammatory cytokine expression during DSS-induced colitis. As expected from the clinical signs, inflammatory cytokines were attenuated in the MLN of DSS-treated wildtype mice that received APP compared to controls. However, when DSS-treated αβ T cell-defieicnt mice were treated with APP, they showed an increase in inflammatory cytokines compared to PBS-treated mice. The authors propose that, in the absence of regulatory αβ T cells, the proinflammatory activity of the γδ T cell is observed, leading to increased inflammatory cytokine production.

## Acai Polysaccharides Promote Lung Resistance to Intracellular Bacteria

Skyberg *et al*.[415] sought to characterize the potential therapeutic effects of plant agonists during infection with the intracellular bacterial pathogens *Francisella tularensis* and *Burkholderia pseudomallei*. To this end, RAW264.7 macrophages were treated with either OPCs (APP), Amphotericin B, Securinine, or the plant polysaccharides from Yamoa and Acai prior to infection. AcaiPS and YamPS were able to induce Nitric Oxide (NO) production and *F. tularensis* killing similar to the positive control, LPS. Further experiments showed that human cells respond similarly, however they required the addition of NK cells to induce these responses and showed that bacterial clearance was dependent upon IFNγ production from the NK cells.

To determine if AcaiPS could likewise mediate these protective effects against *F. tularensis in vivo*, survival studies were performed. In these studies, mice were treated with AcaiPS either by oral gavage or intratracheally. Mice treated by oral gavage showed some protection from the disease both when given AcaiPS prophylactically or therapeutically. The average survival over three studies in mice receiving prophylactic AcaiPS was 12/40 (30% survival) compared to 0/30 for the untreated (0% survival). In two experiments, mice were also administered therapeutic AcaiPS by oral gavage at days 0, +1, and +2. Of these, 4/30 mice survived compared to 0/20 for the untreated mice.

While these results were striking in themselves, since *F. tularensis* infection is normally 100% fatal, the most impressive results were in mice that received AcaiPS intratracheally. Prophylactic treatment with 10µg, 100µg, or 1mg AcaiPS resulted in

survival rates of 40%, 80% and 20% respectively, compared to a 0% survival with the vehicle-treated mice. Therapeutic treatment with AcaiPS for *F. tularensis* infection was also measured. In these experiments, mice given AcaiPS immediately after infection, after 24hours, or at 48hours post-infection had survival rates of 70-80%, 60%, and 33% respectively.

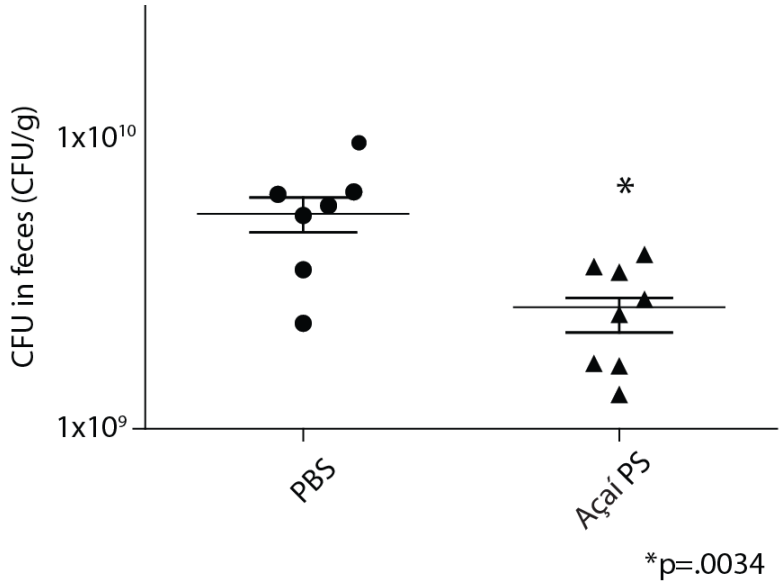
To measure the effects of AcaiPS on bacterial dissemination, Skyberg *et al*.[415] next used the similar pathogen, *B. pseudomallei*. In *B. pseudomallei*-infected mice, bacteria burdens from the spleens, lungs and liver were compared between AcaiPS- and vehicle-treated mice. Treatment with either 100μg or 1mg AcaiPS reduced lung burden by 10,000fold. Perhaps more important for disease progression, bacterial dissemination into the liver and spleen was below the limits of detection in AcaiPS-treated mice.

Successful clearance of these infections is highly dependent upon the Th1 cytokines, IFNγ and IL-12.[416,417,419] Therefore, in the final series of experiments, Skyberg *et al.*[415] elaborated upon the apparent induction of Th1 responses in the lungs of mice treated with AcaiPS. First, they showed that both γδ T cells and NK cells, expressing the Th1 transcription factor, T-bet$^+$, were increased in AcaiPS-treated versus vehicle-treated mice. Next, they showed that mAb-mediated depletion of either NK cells or IFNγ reduced the survival of AcaiPS-treated mice to that comparable to PBS.

This study elaborates upon our previous observation of an increased IL-12 production and lung remodeling by AcaiPS[9] to show that it also increases the number of T-bet$^+$ NK and γδ T cells in the lung. More importantly, this study shows the efficacy of these induced Th1 responses in treating, both therapeutically and prophylactically, disease

caused by the intracellular bacterial pathogens, *F. tularensis* and *B. pseudomallei*. Other experiments are underway to determine the effectiveness of this polysaccharide in protecting against other intracellular pathogens.

Figure 7.2 Therapeutic Treatment of Mice with *Salmonella* Typhimurium-Induced Enterocolitis with AcaiPS Reduced Bacterial Counts in the Feces.




BALB/c mice were pre-treated with streptomycin, then infected with a streptomycin-resistant strain of *ST* ($1x10^6$ CFU/mouse) and 8h later injected with 3mg of AcaiPS or vehicle only (PBS). At 24h post-infection, fecal pellets were collected for assessment of bacterial load on LB agar containing streptomycin (CFU/g feces). Thin bar indicates mean, error bars represent SEM. p-value by Student's T test.

Preliminary data performed in collaboration with Jodi Hedges shows that AcaiPS may also impact intestinal infections, in particular, *Salmonella enterica* serovar Typhimurium. Mice infected with *S.* Typhimurium, using the streptomycin model for enterocolitis, had fewer bacterial counts when treated therapeutically with AcaiPS compared to controls (Figure 7.3). These data suggest that the unique ability of AcaiPS to stimulate TLR4 and Dectin-1 responses could have therapeutic applications in numerous infections.

# CHAPTER EIGHT

# CONCLUSIONS

## Summary of OPC and AcaiPS Responses

Plant products induce numerous effects on the innate immune response, yet there exists a divide between characterizing these immune responses and detailed analyses of the mechanisms for these responses. Studies identifying the mechanisms by which plant products activate the Nrf2 and LR67 pathways have greatly advanced our understanding of the processes some plant products utilize to mediate their effects, thus improving the therapeutic potential of these agonists. The studies herein begin to similarly characterize the mechanisms leading to the activation of innate responses by OPCs and plant polysaccharides. These studies will likewise aid in improving the understanding of the innate immune responses generated by these compounds when applied to *in vitro* culture or *in vivo* disease settings.

The innate responses induced by Acai polysaccharides and OPCs are very different. While AcaiPS activates a robust Th1 response, OPCs generate a more subtle response characterized by GM-CSF production and transcript stabilization. To further clarify these differences, we tested AcaiPS in the BE5B reporter line and found that it does not alter transcript degradation (data not shown). This suggests the mechanisms of OPC-mediated immune activation are separate from those mediated by AcaiPS. The activation profile of AcaiPS is also much more overt than OPCs, and lead to the generation of a Th1-like response from innate immune cells. The innate stimulatory properties of these two

compounds are compared in Table 8.1. Although their responses do overlap to some degree, such as the use of syk for their activity, the bulk of the responses are very different.

Table 8.1 Comparison of Innate Immune Responses Induced by Acai Polysaccharides and OPCs

| | AcaiPS | OPCs |
|---|---|---|
| *In vivo* | | |
| Lung disease | Induce a Th1 response that is protective against *F. tularensis* | Protect against viral challenge via type I IFN |
| intestinal | Decreased burden of *S.* Typhimurium | Reduce inflammation due to DSS colitis via αβ T cells |
| systemic | LPS-like tolerance (data not shown) | Increased Neutrophil recovery after antibody-mediated neutropenia |
| peritonitis | Recruited neutrophils | Recruited neutrophils |
| *In vitro* | | |
| Purified γδ T cells | Minimal direct activity. Requires monocytes for optimal response | Directly activates and primes for proliferative responses with IL-2 secondary culture |
| MOLT-14 transcript stabilization | No change | Stabilizes *GMCSF 3'UTR* |
| PBMC cytokines | Robust TNFα, IL-1β, IL-6 and IL-10 production | Production of GM-CSF, MIP1-α, IL-1β, IL-6, and IL-8 |
| Syk kinase activated? | Yes, in the presence of serum | Probably, syk inhibition prevents BE5B stabilization |
| TLR-mediated? | Yes | No. CD14 or TLR4 antibody blockade does not alter OPC-induced stability (not shown) |
| Dectin-1 binding | Yes | Not tested |
| toxicity | >500µg/mL | 20-200µg/mL |

The results of this dissertation provide information on the signaling mechanisms induced by OPCs and by AcaiPS. Although there remains work to be done in describing both of these innate agonists, we currently have enough data to compile working models

for each agonist. In regard to OPCs, many components of the signaling pathway involved in transcript stability are defined, yet the receptor(s) mediating this response still elude us (Chapters 3 and 4). Our working model addresses the complexities of inflammatory and anti-inflammatory responses induced by OPCs and links syk as a central mediator for these innate immune cell agonists.

Alternatively, AcaiPS is shown to activate both the TLR4 and Dectin-1 receptors, and this combined signaling response promotes a Th1 cytokine response (Chapters 5 and 6). While responses as a result of this dual receptor engagement are being worked out by others using multiple ligands or polysaccharides with weak TLR4 stimulatory activity,[75,77,423,424] the research presented herein demonstrates that AcaiPS may function as a single, robust agonist for their responses. Our current working models for OPCs and AcaiPS, generated from the data provided within this dissertation, will next be presented in separate sections in this concluding chapter.

## The Discrepancies in OPC-Mediated Responses Explained by a Syk-Dependent Model

The *in vitro* and *in vivo* responses to OPCs are very conflicted. *In vitro*, there are studies showing that OPCs promote the generation of inflammatory responses[1-3,226,227,236,237,248-250] while also generating responses that inhibit TLR4-mediated activation (See Chapter Two, Table 2.7). In addition to these *in vitro* effects described previously, we observe a similar phenomenon with OPC treatment *in vivo*. On one hand, when mice receive OPCs on a short-term, limited-dose basis, an inflammatory phenotype develops.

These results are observed with the antiviral responses, neutrophil recruitment, and neutrophilia responses described in the previous chapter. Alternatively, an anti-inflammatory phenotype is observed when mice are provided high doses of OPCs over a term of more than a week. These high-dose/long-term treatments protect against inflammatory diseases including DSS-induced colitis as described by Skyberg *et al*[240] in the previous Chapter. In addition, high-dose/long-term OPC treatment protects in a mouse model for Multiple Sclerosis (EAE), as is currently being detailed by Eduardo Huarte *et al.* (manuscript in preparation). A similar, OPC-mediated protection against EAE, was observed by Miyake *et al.* after repeated i.p. injection with many, but not all, OPC-rich extracts. This protection was associated with suppressed Th1-like responses.[425] Explanations for how these discrepancies in inflammatory and anti-inflammatory responses might occur have eluded the OPC research community, since their first descriptions in the early 2000's.[327,426] Our observation that syk is involved in OPC-mediated transcript stability may finally shed light upon how both of these responses occur.

In spite of the fact that syk plays a central role in many immune responses, its activation mechanisms remain poorly understood. Classically, syk is activated by src-activated immunoreceptor tyrosine activation (ITAM)-containing receptors; however, recent evidence indicates that syk can also autophosphorylate and that ERK can also mediate syk serine phosphorylation.[427,428] These observations that syk can autophosphorylate and that other kinases may affect its activity add an additional layer of complexity to this kinase. These observations are supported by limited observations of

src-independent activation, yet the mechanisms of this response remain poorly understood.[429,430]

Under the classic activation sequence for syk, the ITAM-containing receptors are first activated/phosphorylated by upstream src-family kinases before activating syk. Therefore these two kinases systems are closely linked, with syk activation occurring in a subset of src-activated pathways. The classic receptors mediating syk activation are the Fc-like receptors, these include the FcRs, the Dectin-2 family of receptors, the Triggering Receptor Expressed on Myeloid cells (TREM) family of receptors, and others.[336] Other receptors with ITAM-like domains also use this kinase to modulate cell responses. These include Dectin-1, the selectins, integrins, and IL-3R.

In addition to the typical responses associated with Src activation [FAK/Pyk2 activation (cytoskeletal rearrangement) and calcium flux], ITAM-containing receptors also activate syk. Syk leads to, and is associated with, multiple signaling pathways. The sum of these responses are reviewed in detail elsewhere.[336,431] Of importance to potential mechanisms for OPC-mediated responses is the syk-mediated activation of responses that both promote and attenuate TLR-mediated signaling.

Syk can promote innate inflammatory responses by activating ERK and NFκB. The phenotype induced by this signaling cascade is very similar to what we observe with OPCs, a modest change in activation and cytokine production as well as ERK activation. This would also explain the added responses to TLR4 ligation during co-culture with OPCs that is observed by Kenny *et al.*[236] since syk and TLR activation of NFκB is additive.[77]

In contrast to these inflammatory effects from syk signaling, there are numerous reports of OPC pre-treatment attenuating TLR4-mediated responses.[234,251,252,257] These observations occur from OPC-pretreated, but not co-treated cells,[236] so there is likely a change in the phenotype of the cells that mediate this attenuated response. This hypothesis is supported by the reduced NFκB activation potential in OPC-pretreated cells.[234,251-253,257] The activation of syk by OPCs may explain these observations. Syk activation attenuates TLR-mediated signaling via numerous mechanisms (Figure 8.1). First, syk-mediated signaling promotes IL-10 production via ERK.[432] This anti-inflammatory cytokine attenuates cell responses and can serve as a negative feedback for TLR-mediated responses.[336] Secondly, syk/src can activate the phosphatase calcineurin,[433] which may lead to dephopshorylation of TLR-associated adaptor molecules.[434] Finally, a recent report by Han *et al*.[107] shows that syk can directly phosphorylate the TLR adaptor molecules TRIF and MyD88. This phosphorylation leads to ubiquitination, degradation of the adaptor molecules, and subsequently prevents TLR signals from being generated.[107] In addition to syk, if the OPCs follow the classic syk activation profile mediated by src kinases, src kinases are also able to attenuate TLR-mediated responses.[336] One or more of these TLR-attenuating responses may explain how OPCs, which we show activate syk, induce both pro-inflammatory responses and attenuate TLR-mediated inflammation.

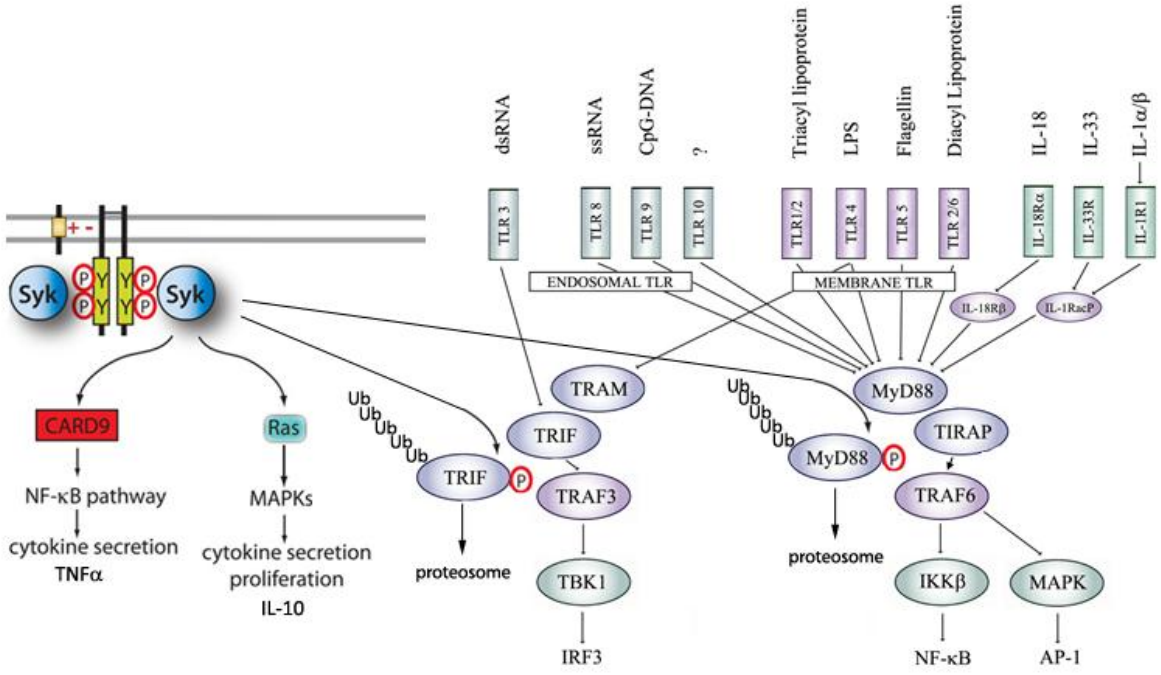
There are currently a very large number of syk inhibitors that are being pursued for their potential to suppress many immunological effects and thus their potential pharmaceutical use.[435-438] Kinases, such as syk, are a valuable target for immunotherapy

because they function as on/off switches and have very defined roles in the immune system. Currently, modulators of syk activity focus on inhibition. The ability of OPCs to promote syk activation may also have numerous, opposing effects such as in infectious diseases or in neutropenia as shown in the previous chapters. As discussed, these effects are both pro- and anti-inflammatory in nature. Therefore, OPCs may supplement currently pursued kinase inhibitors, to activate syk and more acutely direct kinase activity. Further analysis of the syk-activating pathways and the specificity of OPCs may identify unique applications for these compounds in medicine.

## A Model Describing the Robust Response to AcaiPS via TLR4 and Dectin-1 Signaling

The characterization of AcaiPS as a potent therapeutic for tularemia by Jerod Skyberg[415] demonstrates a compelling application for this agonist. The effects of this response may be explained by our earlier observation that AcaiPS activates a Th1-type response when delivered to the lung.[9] We propose this robust response is due to the activation of both the TLR4/TRIF/MyD88 and the Dectin-1/syk pathways. Our data indicate that the TLR4-dependent response is mediated by acetylation of the polysaccharide. Likewise, the recognition of polysaccharides by Dectin-1 is dependent upon β-glucan linkages, and we identified these linkages in AcaiPS. From these results, we are able to develop a model for the robust responses induced by this TLR4/Dectin-1 agonist (Figure 8.2).

Figure 8.1 The Activation of Syk may both Promote and Attenuate TLR-Mediated Responses.




Syk promotes NFκB activation and MAPK activity. This will lead to a moderate inflammatory response, similar to NFκB activation by TLR agonists. Although this will amplify short term responses by TLR agonists, the ability of syk to mediate degradation of the TLR adaptor molecules, TRIF and MyD88, will diminish these responses. Part of the figure was adapted from Lowell.[336]

There are previous reports describing co-signaling with both of these receptors due to their co-ligation during many fungal infections.[77,273,423,424] Although these infections likely do not mimic the response to AcaiPS, we can draw some preliminary conclusions as to how AcaiPS might modulate the innate immune response. As an example of previous studies, both Dectin-1 and TLR4 receptors, but neither TLR2 receptor nor MD2, are necessary for corneal protection against *A. fumigatus* infection. The signaling of other *C. albicans* and *C. neoformans* polysaccharides likewise utilize TLR4.[273,274]

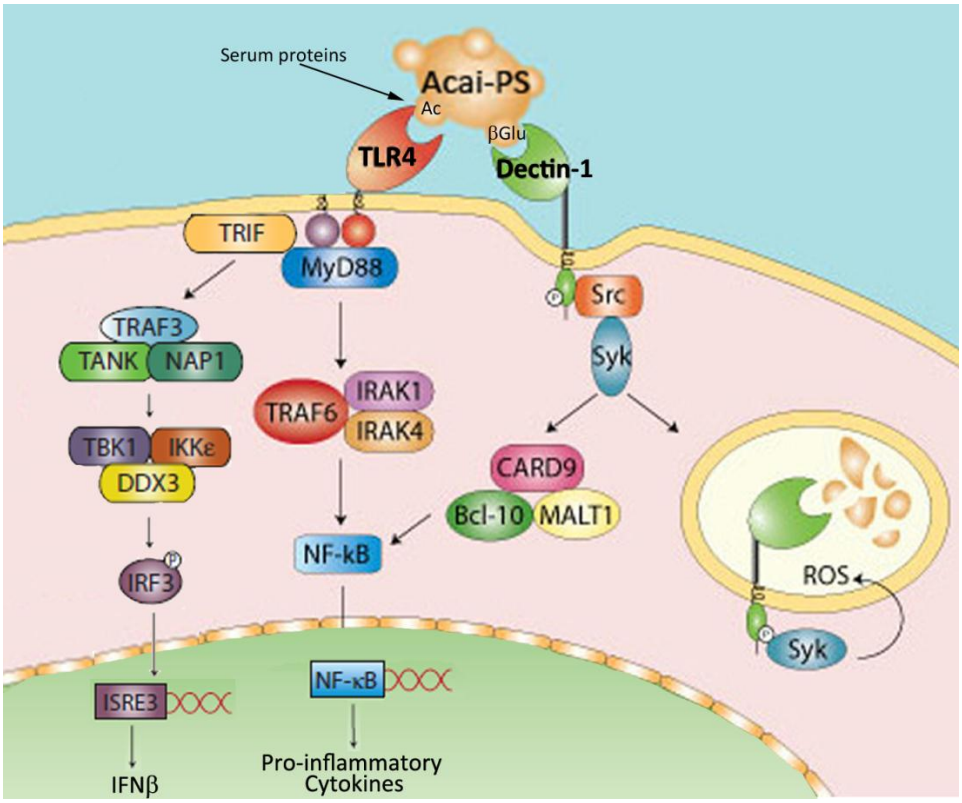
Although these studies with other fungal polysaccharide TLR4 agonists did not look at MD2 dependence, the study with *C. neoformans* polysaccharides identified a dependence for CD14 as an adaptor molecule.[274] The results of these studies indicate that the response to fungal polysaccharide stimulation is often diminished in comparison to LPS. For example, *C. neoformans* TLR4 ligation does not lead to TNFα or MAPK activation, and *A. fumigatus* TLR4 ligation does not affect cellular infiltration. A direct comparison of *C. albicans* was not performed. In contrast to these studies with fungal polysaccharides, the TLR4-mediated response to AcaiPS requires MD2 for an optimal response and AcaiPS is particularly effective in activating cytokine production in human PBMCs, even when syk is inhibited (Figure 6.7). Whether the MD2-mediated response is more "potent" than that of the other polysaccharides with TLR4-stimulating activity remains to be directly compared, but it does appear to induce a more robust production of TNFα than zymosan alone does (Figure 6.7) indicating distinct responses to these polysaccharides may exist.

Previous reports also show that coordinated Dectin-1 and TLR4 signaling promotes a more robust generation of IL-12 than either signaling response alone.[75] The co-activation of both of these receptors leads to the prolonged degradation of the NFκB inhibitor, IκB,[77] leading to a sustained and robust cytokine response.[75,77,424] Much of this response may be due to Dectin-1, which controls of NFκB activation status of the cell via the activation of Raf-1.[75] In addition to these changes to NFκB activity, the activation of Dectin-1 promotes a Th1 response,[75] the activation of the inflammasome[79], endocytosis, and ROS production.[439]

In spite of the phagocytic responses and the shift toward Th1 responses that are generated by Dectin-1 ligation, Dectin-1-mediated cytokine production is relatively moderate.[75,424] This explains how Dectin-1-mediated responses are relatively subdued in the absence of secondary stimuli.[440] Without the activation of nearby cells via cytokine production, exclusive Dectin-1-mediaited responses are localized. However, when combined with TLR4, Dectin-1 promotes a Th1 centric response, which classically promotes phagocytic responses. At the same time, TLR ligation promotes NFκB activation and the production of large amounts of cytokine. This cytokine production is then directed by Dectin-1 and Raf-1 toward an increased production of IL-12. IL-12 then further promotes a Th1-centric cytokine response that will continue to feed into the promotion of a more comprehensive Th1-directed response.

This model (Figure 8.2) is of particular interest in regards to lung responses. The limited responses to TLR4 and Dectin-1 signaling alone serve as a mechanism to protect against overt inflammation in the presence of innocuous antigen, such as dust and aerosolized cellular debris. This protection from overt inflammation comes into play in the lung tissues were inflammation is tightly regulated. As described previously, LPS responses are attenuated in the lung by surfactant proteins.[441] This may also be true for some Dectin-1-mediated responses.[442] Studies describing the lung inflammatory responses to LPS, Dectin-1 agonists, or combinations thereof show how dramatic the ligands, when combined but not individually, mediate inflammatory responses.[443] These observations may explain why AcaiPS, containing both TLR4 and Dectin-1 binding regions, can activate such a robust response in the lung.

Figure 8.2 Proposed Model for the Activation of Innate Immune Pathways via AcaiPS.




Current and Future Directions

A primary question remaining from these studies is how OPCs and AcaiPS can mediate such different responses. To understand this, we must further characterize the receptors and pathways involved. Since both OPCs and AcaiPS rely on syk, and there is some link to MyD88 for both of these responses, how then do they diverge? It is unlikely to be as simple as the activation of TLR4 by AcaiPS since LPS co-stimulation, as reported by others, is only moderately increased by OPCs during co-treatment and, in fact, is attenuated after OPC-pretreatment. Clearly, the activation of syk plays only a

partial role in both of these responses, and a more detailed understanding of the pathways engaged by these compounds must be further elucidated.

One large hurdle in understanding OPC-mediated changes to innate cells is the vast amount of conflicting data as to their inflammatory or anti-inflammatory responses. The identification of ERK and, in particular syk, as required kinases for OPC-induced transcript stability (Chapters 3 and 4) provides some evidence as to the mechanism by which this occurs and allows us to generate the syk-dependent model described previously. In addition to these observations, data not shown here indicate that at least some OPC-mediated responses are dependent upon the TLR adaptor, MyD88 (peritonitis, Jill Graff and Brett Freedman). MyD88/NFκB signaling can in turn suppress its own responses. Therefore, the observation that MyD88 signaling occurs during some OPC-mediated events may also explain the previous reports by others of suppressive responses to LPS.

To elaborate upon the observations showing a requirement for MyD88 in OPC-mediated peritonitis, I have tested numerous MyD88-dependent ligands (LPS, IL-1β, IL-18, IL-33, Lipoteichoic acid, Peptidoglycan, PolyI:C, LPS, and imiquimod) for their ability to induce stability in the BE5B cell line. Although IL-1β can induce *GMCSF* expression in the MOLT14 line (data not shown), thus far I have not observed an increase in transcript stability from any these TIR ligands (data not shown). These preliminary data need to be repeated and include additional TIR agonists, but this observed lack of a response could be due to the lack of appropriate receptors in the MOLT14 cell line. Alternatively, the lack of response could mean that OPC-induced peritonitis is

independent of the mechanisms inducing transcript stability. To address this, I am developing a siRNA transfection protocol to deliver siRNA for *MYD88* to BE5B cells with the goal of determining if, by directly reducing MyD88 expression, OPC-mediated stabilization of transcripts is affected. I also intend to determine if MyD88 expression is altered during OPC-treatment. In the event that syk mediates degradation (See Figure 8.1), we would expect to see decreased MyD88 expression in OPC-treated MOLT14 cells and that this decrease could be attenuated using the syk inhibitor.

Unlike a typical TLR agonist, OPCs induce a much more reserved and atypical activation profile, emphasizing priming over overt responses. Although the MAPK ERK is induced during OPC treatment, p38 is not (Chapter 3). This is in contradiction to the typical robust activation of p38 and the moderate activation of ERK as a result of TLR signaling.[444,445] The stabilization of GFP in the BE5B reporter is dependent upon syk. There are reports of syk activation during TLR signaling, yet this may be mediated indirectly by CD14/syk.[61] In addition to its activation during TLR signaling, syk is classically activated by ITAM-containing receptors via src-family kinases.[336] Therefore, along with testing MyD88-dependent receptors for their role in OPC-mediated transcript stability, I also plan to determine if activated ITAM-based receptors might also be involved. To this end, I plan to include small-molecule inhibitors for the src-family kinases (PP1, PP2, and/or A419259) when screening for OPC-mediated stability using the BE5B cell line.

Syk is also activated by AcaiPS (Chapter 6). This most likely occurs via Dectin-1-mediated responses. In addition to Dectin-1, we also identified TLR4 as a co-receptor for

this plant polysaccharide. Other reports have described both TLR4 and Dectin-1 signaling properties from plant polysaccharides, but this is the first to show a polysaccharide with the potential to robustly stimulate both innate receptor pathways. The combined responses to both TLR agonists and Dectin-1 are important in the recognition and clearance of fungal and mycobacterial pathogen,[273,407,423] and their co-signaling properties are well described for TLR4[77,423,424] as well as for TLR1/2 and TLR9.[407,446-448] These studies emphasize the contribution of syk in both promoting and attenuating TLR responses.

One question remaining is whether AcaiPS binding to Dectin-1 results in a functional response or whether another receptor may be involved. The ability of Dectin-1 to bind to β-glucans is well documented; however, there remains a large amount of literature showing fungal polysaccharides must be in particulate form for Dectin-1 recognition. AcaiPS is soluble, therefore these results seem to be a departure from fungal polysaccharide recognition. Yet there are other soluble plant polysaccharides that can bind and signal via Dectin-1. β-glucan linkages are identified for soluble β-glucans from plants including barley,[21] brown seaweed (laminarin), AcaiPS, and Yamoa PS (Yamoa blocks anti-dectin-1 mAb binding, data not shown). Although laminarin is generally considered an inactive β-glucan that binds Dectin-1 but does not signal, the activation of innate immune responses by the others are described. [4,9,21] This response is well shown with the barley β-glucan by Dectin-1 transfection of 293T cells as discussed previously.[21] The ability of plant polysaccharides to signal via Dectin-1 would indicate that the

solubility requirements for fungal polysaccharides may not apply to at least some plant polysaccharides.

One way in which plant polysaccharides are structurally different from fungal polysaccharides is the observation that many, including AcaiPS, bind TLR4.[261,268,269,278] TLR4/CD14 in these, as well as many mushroom polysaccharides,[261,409] may act as a scaffold for endocytosis[61] and thus Dectin-1 recognition. Is this then a common feature for plant polysaccharides? Many are shown to have acetylated residues similar to AcaiPS and *Klebsiella* capsular polysaccharide.[279,449-451] A final piece of evidence supporting the formation of a Dectin-1/TLR4 complex is the observation that Dectin-1 requires Mac-1 for neutrophil-mediated endocytosis.[72] This supports the possibility of endocytic receptors mediating Dectin-1 activation and indicates that Dectin-1 signaling is a result of phagocytic receptor cooperation. It is therefore possible that TLR4/CD14 signaling may fulfill the same role for plant polysaccharides.

The observation that TLR4 responses to AcaiPS are attenuated upon de-acetylation of the polysaccharide may provide a key element in how TLR4 recognizes AcaiPS and other polysaccharides. Many polysaccharides, such as laminarin, do not contain acetylated polysaccharides, yet the per-acetylation of polysaccharides is relatively easily accomplished with acetic anhydride.[452] Future studies could determine if acetylation is able to convert laminarin, or another polysaccharide, to a TLR4 ligand. Furthermore, immunomodulatory polysaccharides, such as zymosan or curdlan, could likewise be modified to determine if this would also activate TLR4. In the event acetylation could induce TLR4 activation in numerous polysaccharides, immunomodulatory

polysaccharides could be customized to generate different responses. Unlike the current vaccine adjuvant MPL, which predominantly engages via TRAM/TRIF, we predict that an acetylated polysaccharide would also signal via MyD88. The potential to generate a functional TLR4 agonist by acetylation of polysaccharides may enable the generation of numerous adjuvants with an improved ability to generate appropriate vaccine responses.

Future studies looking at AcaiPS should also focus on looking at how manipulating the innate response in the lung can be used for benefit in other disease models. In addition to *Francisella* and *Burkholderia* infections of the lung, many other lung pathogens are readily controlled by Th1-assocaited responses. These include fungal[285] and bacterial[453] infections of the lung tissue. Finally, it may be of value to test other diseases that can be treated with Th1 therapy, in the context of AcaiPS-induced Th1 cytokines.

In conclusion, numerous plant products are described with very diverse functions in activating the innate immune system. Many, such as the Nrf2 and LR67 agonists, promote anti-inflammatory responses. Alternatively, the OPCs and AcaiPS stimulate a more pro-inflammatory immune response, yet both of these are very different. Whereas OPCs stabilize transcripts and lead to a priming-like phenotype, AcaiPS is much more overt in its ability to activate the innate immune system and generates a Th1-centric response from the innate immune system. The ability to stimulate the innate immune response differently may indicate applications for each compound separately or in tandem as therapeutics or vaccine adjuvants. However, before either can actually be used in a clinical setting, studies detailing receptors, relevant structures for activity, and

biochemical pathways stimulated by each must be further delineated. The studies performed as part of this dissertation contribute to our understanding of these plant products and will assist the future development of these compounds or similar molecules as therapeutic agents.

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# APPENDICES

# APPENDIX A:

## LIST OF ABBREVIATIONS AND ACRONYMS

**67LR** 67kDa Laminin receptor
**APP** Apple polyphenol
**AURE** AU Rich element
**BALB/c** mouse strain
**BALF** Bronchoalveolar lavage fluid
**BE5B** MOLT-14 reporter line expressing GFP3'UTR-CSF2
**β-glucan** β(1,3)glucan-containing polysaccharide
**C57BL/6** mouse strain
**CD** Cluster of Differentiation
**CFA** Complete Freund's Adjuvant
**CFSE** Carboxyfluorescein succinimidyl ester
**CLR** C-type Lectin Receptor
**CXCL1** CXC family chemokine Ligand 1
**CXCR2** CXC family chemokine Receptor 2
**DAMP** Damage-Associated Molecular Pattern
**DETC** Dendritic-like Epithelial T cell
**DSS** Dextran Sodium Sulfate
**ECM** Extracellular Matrix
**EGCG** Epigallocatechin Gallate
**ELISA** Enzyme-linked Immunosorbent Assay
**ERK** Extracellular signal-regulated Kinase
**GAG** Glycosaminoglycans
**GCG** Gallocatechin Gallate
**GM-CSF** CSF2, Granulocyte/Macrophage Colony-Stimulating Factor
**GSE** Grape Seed Extract
**HRP** Horse Radish Peroxidase
**IFN** Interferon
**IGF** Insulin-like Growth Factor
**IL** Interleukin
**JNK** Jun N-terminal Kinase
**KC** see CXCL1
**KGF** Keratinocyte Growth Factor
**LAL** Limulus Amebocyte Lysate
**LBP** LPS-binding protein
**LDL** Low-Density Lipoprotein
**LPS** lipopolysaccharide, endotoxin
**mAb** Monoclonal antibody
**Mac-1** Macrophage-1 antigen 1, CD11b/CD18 heterodimer
**MAPK** Mitogen-activated protein kinase
**MD-2** Lymphocyte antigen 96
**MIP** Macrophage Inflammatory Protein
**MM6** MonoMac-6 cell line
**MMR** Macrophage Mannose Receptor, see MR
**MOLT-14** γδ T cell-like cell line
**MR** Mannose Receptor, CD206
**MyD88** Myeloid Differentiation primary response gene (88)
**NFκB** Nuclear Factor kappa-light-chain-enhancer of activated B cells
**NK** Natural Killer cells
**NLR** NOD-like Receptors
**NOD** Nucleotide Oligomerization Domain
**Nrf2** Nuclear Factor (erythroid-derived 2)-like-2
**OB** Oenothein B
**OPC** Oligomeric Procyanidin
**p38** p38 MAPK
**PAMP** Pathogen-Associated Molecular Pattern
**PB1** Procyanidin B1
**PB2** Procyanidin B2
**PBMC** Peripheral Blood Mononuclear Cell
**PBS** Phosphate Buffered Saline
**PC1** Procyanidin C1
**PRR** Pattern Recognition Receptors
**qPCR** quantitative PCR
**RLR** RIG-like receptors
**RORγt** RAR-related orphan receptor gamma, RORγ2 isoform
**ROS** Reactive Oxygen Species
**Syk** spleen tyrosine kinase
**T-bet** T-box transcription factor TBX21
**TCR** T-cell Receptor
**TF3** Theaflavin digallate
**Th** T-helper cell or their associated cytokine profile
**TIR** Toll/Interleukin-1 Receptor homology domain
**TLR** Toll-like Receptor
**TNFα** Tumor Necrosis Factor-alpha
**TRIF** TIR-domain-containing adapter-inducing interferon-β
**U0126** MEK1/2 inhibitor
**uLPS** ultrapure LPS
**UTR** Untranslated Region
**WC1** Workshop Cluster 1 scavenger receptor

# APPENDIX B:

## ABSTRACTS FROM ADDITIONAL AUTHORED PUBLICATIONS

# Response of gammadelta T Cells to plant-derived tannins

Authors
Holderness J, Hedges JF, Daughenbaugh K, Kimmel E, Graff J, Freedman B, Jutila MA.

Reference
Crit Rev Immunol. 2008;28(5):377-402. Review.

Abstract
Many pharmaceutical drugs arc isolated from plants used in traditional medicines, and new plant-derived pharmaceutical drugs continue to be identified. Relevant to this review, different plant-derived agonists for gammadelta T cells are described that impart effector functions upon distinct subsets of these cells. Recently, plant tannins have been defined as one class of gammadelta T cell agonist and appear to preferentially activate the mucosal population. Mucosal gammadelta T cells function to modulate tissue immune responses and induce epithelium repair. Select tannins, isolated from apple peel, rapidly induce immune gene transcription in gammadelta T cells, leading to cytokine production and increased responsiveness to secondary signals. Activity of these tannin preparations tracks to the procyanidin fraction, with the procyanidin trimer (C1) having the most robust activity defined to date. The response to the procyanidins is evolutionarily conserved in that responses are seen with human, bovine, and murine gammadelta T cells, although human cells show less selectivity. Procyanidin-induced responses described in this review likely account for the expansion of mucosal gammadelta T cells seen in mice and rats fed soluble extracts of tannins. Use of procyanidins to activate gammadelta T cells may represent a novel approach for the treatment of tissue damage and autoimmune diseases.

# Select plant tannins induce IL-2Ralpha up-regulation and augment cell division in gammadelta T cells

Authors
Holderness J, Jackiw L, Kimmel E, Kerns H, Radke M, Hedges JF, Petrie C, McCurley P, Glee PM, Palecanda A, Jutila MA.

Reference
J Immunol. 2007 Nov 15;179(10):6468-78.

Abstract
Gammadelta T cells are innate immune cells that participate in host responses against many pathogens and cancers. Recently, phosphoantigen-based drugs, capable of expanding gammadelta T cells in vivo, entered clinical trials with the goal of enhancing innate immune system functions. Potential shortcomings of these drugs include the induction of nonresponsiveness upon repeated use and the expansion of only the Vdelta2 subset of human gammadelta T cells. Vdelta1 T cells, the major tissue subset, are unaffected by phosphoantigen agonists. Using FACS-based assays, we screened primary bovine cells for novel gammadelta T cell agonists with activities not encompassed by the current treatments in an effort to realize the full therapeutic potential of gammadelta T cells. We identified gammadelta T cell agonists derived from the condensed tannin fractions of Uncaria tomentosa (Cat's Claw) and Malus domestica (apple). Based on superior potency, the apple extract was selected for detailed analyses on human cells. The apple extract was a potent agonist for both human Vdelta1 and Vdelta2 T cells and NK cells. Additionally, the extract greatly enhanced phosphoantigen-induced gammadelta T cell expansion. Our analyses suggest that a tannin-based drug may complement the phosphoantigen-based drugs, thereby enhancing the therapeutic potential of gammadelta T cells.

# APPENDIX C:

## ABSTRACTS FROM CO-AUTHORED PUBLICATIONS

# Antigen-independent priming: a transitional response of bovine gammadelta T-cells to infection

Authors
Jutila MA, Holderness J, Graff JC, Hedges JF.

Reference
Anim Health Res Rev. 2008 Jun;9(1):47-57. Epub 2008 Mar 17. Review.

Abstract
Analysis of global gene expression in immune cells has provided unique insights into immune system function and response to infection. Recently, we applied microarray and serial analysis of gene expression (SAGE) techniques to the study of gammadelta T-cell function in humans and cattle. The intent of this review is to summarize the knowledge gained since our original comprehensive studies of bovine gammadelta T-cell subsets. More recently, we have characterized the effects of mucosal infection or treatment with microbial products or mitogens on gene expression patterns in sorted gammadelta and alphabeta T-cells. These studies provided new insights into the function of bovine gammadelta T-cells and led to a model in which response to pathogen-associated molecular patterns (PAMPs) induces 'priming' of gammadelta T-cells, resulting in more robust responses to downstream cytokine and/or antigen signals. PAMP primed gammadelta T-cells are defined by up-regulation of a select number of cytokines, including MIP1alpha and MIP1beta, and by antigens such as surface IL2 receptor alpha (IL-2Ralpha) and CD69, in the absence of a prototypic marker for an activated gammadelta T-cell, IFN-gamma. Furthermore, PAMP primed gammadelta T-cells are more capable of proliferation in response to IL-2 or IL-15 in the absence of antigen. PAMPs such as endotoxin, peptidoglycan and beta-glucan are effective gammadelta T-cell priming agents, but the most potent antigen-independent priming agonists defined to date are condensed oligomeric tannins produced by some plants.

# 6-methyl-2,4-disubstituted pyridazin-3(2H)-ones: a novel class of small-molecule agonists for formyl peptide receptors

Authors
Cilibrizzi A, Quinn MT, Kirpotina LN, Schepetkin IA, Holderness J, Ye RD, Rabiet MJ, Biancalani C, Cesari N, Graziano A, Vergelli C, Pieretti S, Dal Piaz V, Giovannoni MP.

Reference
J Med Chem. 2009 Aug 27;52(16):5044-57.

Abstract
Following a ligand-based drug design approach, a potent mixed formyl peptide receptor 1 (FPR1) and formyl peptide receptor-like 1 (FPRL1) agonist (14a) and a potent and specific FPRL1 agonist (14x) were identified. These compounds belong to a large series of pyridazin-3(2H)-one derivatives substituted with a methyl group at position 6 and a methoxy benzyl at position 4. At position 2, an acetamide side chain is essential for activity. Likewise, the presence of lipophilic and/or electronegative substituents in the position para to the aryl group at the end of the chain plays a critical role for activity. Affinity for FPR1 receptors was evaluated by measuring intracellular calcium flux in HL-60 cells transfected with FPR1, FPRL1, and FPRL2. Agonists were able to activate intracellular calcium mobilization and chemotaxis in human neutrophils. The most potent chemotactic agent (EC(50) = 0.6 μM) was the mixed FPR/FPRL1 agonist 14h.

# Polysaccharides derived from Yamoa (Funtumia elastica) prime gammadelta T cells in vitro and enhance innate immune responses in vivo

Authors
Graff JC, Kimmel EM, Freedman B, Schepetkin IA, Holderness J, Quinn MT, Jutila MA, Hedges JF.

Reference
Int Immunopharmacol. 2009 Oct;9(11):1313-22. Epub 2009 Aug 9.

Abstract
Yamoa (ground bark of Funtumia elastica tree) is marketed and sold as a dietary supplement with anecdotal therapeutic effects in the treatment of asthma and hay fever. We determined that Yamoa and Yamoa-derived polysaccharides affected innate immunity, in part, by priming gammadelta T cells. Gene expression patterns in purified bovine gammadelta T cells and monocytes induced by Yamoa were similar to those induced by ultrapure lipopolysaccharide (uLPS). In the presence of accessory cells, Yamoa had priming effects that were similar to those of LPS on bovine and murine gammadelta T cells, but much more potent than LPS on human gammadelta T cells. The bioactive component of Yamoa was delineated to a complex polysaccharide fraction (Yam-I). Intraperitoneal injection of Yamoa and Yam-I in mice induced rapid increases in peritoneal neutrophils directed by changes in chemokine expression. In support of a unique agonist found in Yam-I, similar peritonitis responses were also observed in TLR4- and MyD88-deficient mice. Therapeutic treatment with Yam-I resulted in decreased bacterial counts in feces from mice with *Salmonella enterica* serotype Typhimurium (ST)-induced enterocolitis. This characterization of the immune stimulatory properties of polysaccharides derived from Yamoa suggests mechanisms for the anecdotal positive effects of its ingestion and that these polysaccharides show potential for application in innate protection from disease.

# Oligomeric procyanidins stimulate innate antiviral immunity in dengue virus infected human PBMCs

Authors
Kimmel EM, Jerome M, Holderness J, Snyder D, Kemoli S, Jutila MA, Hedges JF.

Reference
Antiviral Res. 2011 Apr;90(1):80-6. Epub 2011 Mar 1.

Abstract
Oligomeric procyanidins (OPCs) have been shown to have antiviral and immunostimulatory effects. OPCs isolated from non-ripe apple peel were tested for capacity to reduce dengue virus (DENV) titers. Similar to published accounts, OPCs exhibited direct antiviral activity. The possibility of enhanced innate immune protection was also tested by measuring and characterizing gene and protein expression induced by OPCs during DENV infection. Treatment of DENV-infected human PBMCs with OPCs decreased viral titers and affected the expression of critical innate antiviral immune products. OPCs enhanced expression of MXI and IFNB transcripts in high MOI DENV infected PBMC cultures, and phosphorylation of STAT2 in response to recombinant type I IFN (IFN I). During low MOI infection, addition of OPCs increased expression of STAT1 transcripts, MHC I and TNFα protein production. Thus, OPCs exhibited innate immune stimulation of cells in DENV-infected cultures and uninfected cells treated with IFN I. While OPCs from a number of sources are known to exhibit antiviral effects, their mechanisms are not precisely defined. The capacity of OPCs to increase sensitivity to IFN I could be broadly applicable to many viral infections and two separate antiviral mechanisms suggest that OPCs may represent a novel, robust antiviral therapy.

# Nasal Acai Polysaccharides Potentiate Innate Immunity to Protect against Pulmonary *Francisella tularensis* and *Burkholderia pseudomallei* Infections

Authors
Jerod A. Skyberg, MaryClare F. Rollins, Jeffrey S. Holderness, Nicole L. Marlenee, Igor Schepetkin, Andrew Goodyear, Steven W. Dow, Mark A. Jutila, and David W. Pascual

Reference
PloS Pathogens. 2012 8(3): e1002587

Abstract
Pulmonary *Francisella tularensis* and *Burkholderia pseudomallei* infections are highly lethal in untreated patients, and current antibiotic regimens are not always effective. Activating the innate immune system provides an alternative means of treating infection and can also complement antibiotic therapies. Screening of a natural compound library revealed that polysaccharides derived from the Acai berry (AcaiPS) have the ability to activate human γδ T cells and monocytes. Consequently, the protective effects of AcaiPS on immunity to *F. tularensis* and *B. pseudomallei* were investigated. AcaiPS enhanced the clearance of *F. tularensis* in murine macrophages in a time-, dose-, and nitric oxide-dependent fashion. AcaiPS also impaired replication of *Francisella* in primary human macrophages co-cultured with autologous NK cells via augmentation of NK cell IFN-γ. Nasal AcaiPS given before or after infection protected mice against type A *F. tularensis* aerosol challenge with survival rates of up to 80%, and protection was still observed, albeit reduced, when mice were treated two days post-infection. Likewise, nasal AcaiPS treatment also conferred protection against pulmonary infection with *B. pseudomallei*. AcaiPS dramatically reduced the replication of *B. pseudomallei* in the lung and blocked bacterial dissemination to the spleen and liver. Nasal administration of Acai PS drove T-bet-dependent enhancement of the Th1 cell responses by NK and γδ T cells in the lungs, while neutralization of IFN-γ totally abrogated the protective effect of AcaiPS against pulmonary *B. pseudomallei* infection. Collectively, these results demonstrate Acai PS is a potent innate immune agonist that can resolve *F. tularensis* and *B. pseudomallei* infections, suggesting this agonist has broad-spectrum immunity against virulent intracellular pathogens.