Dectin-1 Expression is Altered by Fungal Infection, Polymicrobial Sepsis, and Glucan Administration.

Tammy Regena Ozment-Skelton

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Dectin-1 Expression is Altered by Fungal Infection, Polymicrobial Sepsis, and Glucan Administration

A dissertation
presented to
the faculty of the Department of Microbiology
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Doctor of Science in Biomedical Science

by
Tammy R. Ozment-Skelton
August, 2006

Keywords: Dectin-1, Glucan, polymicrobial sepsis, Candida albicans, neutrophil, intracellular trafficking
ABSTRACT

Dectin-1 Expression is Altered by Fungal Infection, Polymicrobial Sepsis, and Glucan Administration

by

Tammy R. Ozment-Skelton

Glucans are fungal cell wall PAMPs that promote survival in polymicrobial and candidal sepsis. Dectin-1 is the primary PRR for glucans. The goals of the present study were to characterize 1) the effects of fungal infection on Dectin-1; 2) the effects of polymicrobial sepsis in the presence and absence of glucan on Dectin-1; 3) the effects of systemic administration of glucans on Dectin-1; and 4) the intracellular trafficking of glucans. Mice were either systemically infected with Candida albicans, or made septic by CLP with and without glucan phosphate (GP) injection, or injected with GP. Flow cytometry was performed to assess cell surface Dectin-1 expression. C. albicans sepsis resulted in an increase in the percentage of Dectin-1 positive (Dectin⁺) blood and splenic leukocytes by increasing the percentage of neutrophils. C. albicans infection increased the percentage of Dectin⁺ splenic T cells. CLP decreased the percentage of highly Dectin-1 positive leukocytes in the blood by decreasing the percentage of Dectin⁺ neutrophils. GP treatment in sepsis further decreased the percentages of Dectinhigh blood leukocytes and Dectin⁺ neutrophils. CLP decreased the percentage of Dectin⁺ splenic leukocytes by decreasing the percentage of splenic macrophages. GP administration to CLP mice further decreased the percentage of Dectin⁺ splenocytes by decreasing the percentage of Dectin⁺ macrophages. Administration of GP resulted in a prolonged decrease in the percentage of Dectinhigh blood leukocytes. The changes in Dectin-1 expression with GP were because of
decreases in the percentage of Dectin$^+$ neutrophils and monocytes. In the trafficking studies, macrophages were incubated with fluorescent labeled glucans and then stained for intracellular organelles and signal transduction molecules. Cells were imaged using confocal microscopy. GP is internalized by clathrin and trafficked to the Golgi apparatus. GP internalization is regulated but not dependent on caveolin-1. GP co-localized with SRA, TLR2, and PI3K/p85. The trafficking of laminarin and particulate glucan is similar. We speculate that loss of cell surface Dectin-1 may be important in the protection conferred by glucans in sepsis. Additionally, intracellular trafficking and interaction with signaling components may be important steps in modulation of cellular function by glucan-pattern recognition receptor complexes.
DEDICATION

I would like to dedicate my work to my husband David Skelton. His patience and support have allowed me to reach my goals.
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I would first like to my profound gratitude to the members of my committee. I am indebted to my advisor Dr. David Williams for my professional development. Not only has he provided me with the tools to do good science, but he has also given me with more educational opportunities than I could ever have hoped. I would like to extend special thanks to Drs. John Laffan and Chuanfu Li for their troubleshooting assistance. When things were not working, they were always willing to help. I am also grateful for the assistance of Dr. John Kalbfleisch. Without his statistical expertise, my data would have no significant meaning. Thank you to Dr. Robert Schoborg for talking me out of the clock tower and Dr. John Schweitzer for his support and friendship.

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CHAPTER 1

INTRODUCTION

The Innate Immune System

The primary job of the mammalian immune system is to protect against infectious disease. The immune system can be divided into two interrelated branches, the adaptive or specific branch and the innate or non-specific branch. The adaptive immune response is mediated by T and B lymphocytes that express highly specific receptors against an individual organism. Over the course of infection, new generations of lymphocytes express receptors that are even more specific to the pathogen by mutation of the genes for the receptors and selection of the cells expressing receptors that best recognize the pathogen. The innate immune system on the other hand consists of a myriad of barriers and cellular responses that are not specific to the offending organism. The cells of the innate immune system are integral to a protective immune response. They consist of phagocytic and non-phagocytic cells and are often found at locations of pathogen entry. These cells not only engulf and destroy pathogens, but they are also responsible for mediating the inflammatory response by producing mediators (cytokines and chemokines) that attract and activate immune cells. These responses are immediate, and therefore the innate immune system is the first line of defense against invading pathogens. Additionally, the innate immune system plays an important role in the regulation of the adaptive immune response by production of cytokines and chemokines and presentation of antigen. The resolution of most infections requires the activity of both the innate and adaptive immune systems.
Pattern Recognition Receptors

The cells of the innate immune system, including monocytes, macrophages, neutrophils, dendritic cells, and endothelial cells, are the first cells to respond to invading pathogens. These cells recognize pathogens by virtue of evolutionarily conserved germline encoded receptors known as pattern recognition receptors (PRRs) (Gordon 2002; Akira and Hemmi 2003). These receptors bind to molecules unique to and necessary for the survival of microbes known as pathogen associated molecular patterns (PAMPs) (Gordon 2002; Akira and Hemmi 2003). Many PRRs have been found to also bind to endogenous ligands that are frequently molecules released by damaged host tissues (Johnson et al. 2002; Tsan and Gao 2004). The interaction of PRRs with their ligands is rapid, specific, and gives rise to a diverse array of innate immune responses that are directed at controlling and/or eliminating the pathogen and thus protecting the host from infection (Gordon 2002; Akira and Hemmi 2003). The most studied of the families of PRRs are the toll-like receptors (TLR), and the most studied of this family of 11 members is TLR4. The best known PAMP for TLR4 is lipopolysaccharide (LPS), an integral component of gram negative bacteria (Akira and Hemmi 2003). Upon binding LPS, TLR4 mediates a signal cascade that results in the activation of several transcription factors including nuclear factor (NF)-κB (Figure 1) (Akira and Takeda 2004). This transcription factor stimulates the transcription of genes encoding inflammatory cytokines and chemokines (Akira and Takeda 2004). In phagocytes, TLR4 also mediates uptake of LPS (Husebye et al. 2006). Mice that lack TLR4 are unable to mount an effective immune response to LPS (Hoshino et al. 1999). Many of the other TLRs use similar signaling pathways to TLR4 (Figure 1) (Akira and Hemmi 2003; Akira and Takeda 2004).
Dectin-1. Not all PRRs are TLRs. Dectin-1 is one non-TLR PRR. Dectin-1 is the primary PRR for \((1\rightarrow3)/(1\rightarrow6)-\beta\)-linked glucans (Brown and Gordon 2001; Willment et al. 2001; Brown et al. 2002; Taylor et al. 2002; Brown et al. 2003). When it was discovered, however, Dectin-1 was not originally thought to be a PRR (Ariizumi et al. 2000). Dectin-1 was discovered by Ariizumi et al. using cDNA subtractive cloning (Ariizumi et al. 2000) in which mRNAs from a macrophage cell line were compared to those of a dendritic cell (DC) line.
Dectin-1 was found to be one of the mRNAs exclusively expressed by the DC cell line. Upon expressing the protein for Dectin-1, the amino acid sequence for Dectin-1 was discovered (Ariizumi et al. 2000). As the then unknown protein contained a carbohydrate recognition domain that was homologous to those found in C type lectins, it was designated DC-associated C-type lectin-1 or Dectin-1 (Ariizumi et al. 2000). Further studies by the Ariizumi group determined that the expressed protein would bind to an unknown ligand on T cells. Binding of Dectin-1 to activated T lymphocytes stimulated replication. Therefore, the authors proposed that Dectin-1 was a co-stimulatory molecule for T lymphocytes (Ariizumi et al. 2000). Brown et al. later discovered that Dectin-1 is also a PRR for glucans by screening a macrophage expression library with zymosan, a fungal cell wall extract that is primarily composed of glucan (Brown et al. 2001). In this study, Brown et al. determined that Dectin-1 transfected cells would bind to free glucans and to glucans present in the fungal cell wall (Brown et al. 2001). They also determined that the glucan binding site is independent of the site that binds to and stimulates T lymphocytes (Brown et al. 2001). These pioneering studies have resulted in an explosion of activity in the study of Dectin-1.

Dectin-1 is a type II transmembrane receptor (Brown and Gordon 2001; Brown et al. 2003; Gantner et al. 2003; Rogers et al. 2005). Extracellularly it contains a single C-type lectin-like carbohydrate recognition domain and 1 of the 2 alternatively spliced isoforms contains a cysteine-free stalk region (Figure 2) (Ariizumi et al. 2000; Willment et al. 2001; Willment et al. 2005; Heinsbroek et al. 2006). The lack of cysteine residues in the stalk or the lack of a stalk region suggests that Dectin-1 does not dimerize (Heinsbroek et al. 2006). Dectin-1 has a tyrosine based activation motif (ITAM) in the cytoplasmic tail (Figure 2) (Brown and Gordon 2001; Willment et al. 2001; Brown et al. 2002; Taylor et al. 2002; Brown et al. 2003; Gantner et al. 2003). This motif is required for cellular activation (Brown et al. 2003; Gantner et al. 2003). Upon binding glucan, phosphorylation of tyrosine residues present in the ITAM result in the generation of signal cascades (Gantner et al. 2003; Underhill et al. 2005). These cascades are
Figure 2  Structure of full length murine Dectin-1 (A) and the truncated splice variant (B) (Adapted from Brown et al. 2003)

currently under investigation; however, phosphorylation results in the interaction and activation of SH2-domain containing protein spleen tyrosine kinase (SYK) (Figure 3) (Rogers et al. 2005; Underhill et al. 2005). SYK then mediates the induction of the respiratory burst, a microbial killing mechanism, in macrophages (Figure 3) (Rogers et al. 2005; Underhill et al. 2005). Dectin-1 also mediates a signaling cascade via TLR2, another member of the TLR family (Figure 3) (Brown et al. 2003; Gantner et al. 2003). This interaction results in NF-κB activation and tumor necrosis factor (TNF) α production (Figure 3) (Brown et al. 2003; Gantner et al. 2003). Finally, Dectin-1 is also able to mediate an inflammatory response to glucans by stimulating NF-κB activity and TNFα production in a TLR2 independent manner; however, this pathway is poorly understood (Figure 3) (Brown et al. 2003).
Dectin-1 is expressed by monocytes, neutrophils, macrophages, and, at lower levels, by dendritic cells and subpopulations of T cells (Willment et al. 2001; Brown et al. 2002; Taylor et al. 2002). The amount of Dectin-1 expressed by macrophages is influenced by tissue location as well as activation status (Taylor et al. 2002; Willment et al. 2003). Alveolar macrophages, which are present at a point of pathogen entry, express high levels of Dectin-1, as do inflammatory macrophages (Taylor et al. 2002). Dectin-1 expression is altered by cytokines and microbial products (Willment et al. 2003). Granulocyte monocyte colony stimulating factor (GM-CSF) enhances Dectin-1 expression and function in activated macrophages, whereas interleukin (IL)-10 reduces Dectin-1 expression by macrophages (Willment et al. 2003). LPS
also reduces Dectin-1 expression by macrophages (Willment et al. 2003). Whether other cytokines and chemokines or microbial products will have similar effects has not been explored.

Dectin-1 will bind free glucans as well as whole *Candida albicans* and *Saccharomyces cerevisiae* cells in a glucan dependent manner and, upon binding, mediates rapid internalization of the Dectin-1/glucan complex (Brown and Gordon 2001; Willment et al. 2001; Brown et al. 2002; Taylor et al. 2002; Brown et al. 2003; Herre et al. 2004). Internalization is not required for signal generation, but the type of signal generated by Dectin-1 may depend on internalization (Brown et al. 2003; Herre et al. 2004). When Dectin-1 is internalized with a biologically active glucan, glucan phosphate, Dectin-1 is degraded, and new receptors must be synthesized prior to cell surface expression (Herre et al. 2004). When Dectin-1 is internalized with an inactive glucan, laminarin, the Dectin-1 is rapidly recycled to the cell surface (Herre et al. 2004). The differences in trafficking may be related to differences in activity.

**Pathogen Associated Molecular Patterns**

PAMPs are molecules found exclusively in microbes. They are integral to microbial survival and are, therefore, not alterable by the organism. These characteristics make PAMPs ideal targets for the mammalian immune system. Microbial restricted carbohydrates, lipids, nucleic acids, and proteins can be PAMPs. LPS is one of the most studied PAMPs of Gram negative bacteria, but most microbes have many PAMPs. Gram positive bacteria contain lipoproteins, peptidoglycan, and lipoteichoic acid which are TLR2 ligands (Akira and Hemmi 2003; Akira and Takeda 2004). Viral genetic material can also act as a PAMP. CpG DNA is a ligand for TLR9 and double stranded RNA is a ligand for TLR3 (Akira and Hemmi 2003; Akira and Takeda 2004). Fungi have a variety of PAMPs in their cell walls including mannan, which binds to the mannose receptor and to DC-SIGN (Cambi et al. 2003; Allavena et al. 2004), and glucans, which are ligands for Dectin-1 (Brown and Gordon 2001; Willment et al. 2001; Brown et al. 2002; Taylor et al. 2002; Brown et al. 2003).
(1→3)-β-D-glucan. Glucans are a major cell wall component of both pathogenic and saprophytic fungi, some bacteria, and plants (Chauhan et al. 2002; Williams et al. 2003). They are polymers of glucose that can exist as a non-branched (1-3)-β-linked backbone or as the backbone with (1-6)-β-linked branches (Ensley et al. 1994; Lowman et al. 1998) either in a single helix or triple helical conformation (Figure 4). Glucans have been reported to be masked within the cell wall of fungi, but are exposed at traumatized areas of the cell wall such as bud scars (Figure 5) (Gantner et al. 2005). They are also released from the fungal cell wall, and increased levels of glucans can be found in the blood of patients with fungal infection (Mori et al. 1997; Nakamura et al. 1998; Hiyoshi et al. 1999). Interestingly, elevated glucan levels are also found circulating in the blood of critically ill patients, regardless of the presence of fungal infection (Mori et al. 1997; Nakamura et al. 1998; Hiyoshi et al. 1999; Digby et al. 2003). Studies suggest that the source of these glucans is the gastrointestinal tract, as glucans have been shown to be absorbed from the gut (Rice et al. 2005b). The consequences of circulating glucans are not known.
A. Candida albicans

Figure 4 Molecular structure of \((1\rightarrow3)/(1 \rightarrow 6)\)-b-D-glucan A. Linear structure of \((1\rightarrow3)/(1 \rightarrow 6)\)-b-D-glucan with the backbone chain (BC), reducing terminus (RT), second from the reducing terminus (SRT), and the non-reducing terminus (NRT) indicated. B. Three dimensional representation of the triple helical conformation of glucan (Adapted from Lowman et al. 2003).

Glucans bind to multiple PRRs including Dectin-1 (Brown et al. 2002; Brown et al. 2003; Gantner et al. 2003), type A scavenger receptor (SR-A) (Rice et al. 2002), and complement receptor (CR) 3 (Xia et al. 1999). They have also been reported to interact with lactosylceramide, a glycosphingolipid that forms lipid rafts (Sato et al. 2006). They are able to activate macrophages (Williams et al. 1983; Williams et al. 1996), activate and attract neutrophils (Williams et al. 1988; Tsikitis et al. 2004; Sato et al. 2006), and stimulate inflammatory cytokine production by activation of NF-\(\kappa\)B (Abel and Czop 1992; Williams et al. 1996; Brown et al. 2003; Gantner et al. 2005). Based upon the presence of glucans in the fungal cell wall (Adapted from Shepherd 1987).
cell wall and their ability to induce an inflammatory response, glucans are considered to be important in the innate immune response against fungal infection (Abel and Czop 1992).

Pure glucans have been shown to be biological response modifiers (Williams et al. 1978; Williams et al. 1982; Williams et al. 1988; Williams et al. 1989; Williams et al. 1991b; Lasarow et al. 1992; Williams et al. 1999b; Cheung et al. 2002; Hong et al. 2004). Glucans were originally thought to be primarily immunostimulants. They have been shown to increase resistance to challenge with bacteria or fungi primarily by their activating effects on macrophages and neutrophils (Williams et al. 1982; Williams et al. 1988). They have also been reported to be potent adjuvants (Williams et al. 1989; Cheung et al. 2002; Hong et al. 2004). Recent studies by Cheung et al. and Hong et al. have demonstrated that administration of glucans enhances the tumoricidal activity of anti-tumor antibodies (Cheung et al. 2002; Hong et al. 2004).

Figure 6 Glucan phosphate administration in mice improves survival in polymicrobial sepsis (Adapted from Williams et al. 1999b)
Glucans also facilitate wound repair by stimulating macrophages and collagen biosynthesis (Portera et al. 1997; Wei et al. 2002). Interestingly, glucans have non-immunomostimulatory effects as well. Administration of glucan phosphate (GP) significantly increases survival outcome in mice subjected to cecal ligation and puncture (CLP) induced polymicrobial sepsis (Figure 6) (Williams et al. 1999b). Glucans have also been shown to reduce cardiac ischemia/reperfusion injury (Li et al. 2003). An excessive inflammatory response contributes to the pathophysiology of both of these conditions (Williams et al. 1999b). Glucans have been found to blunt this response (Williams et al. 1999b). As the first step in the modulation of cellular function by glucans is binding of the glucan by membrane associated PRRs (Mueller et al. 1996; Mueller et al. 2000; Rice et al. 2002), it is likely that the PRRs for glucans play a significant role in both immunostimulatory and immunomodulatory responses.

Differences in size and conformation between different glucans result in different biologic activities and binding affinities for Dectin-1. For instance, GP, a soluble glucan derived from \textit{S. cerevisiae} with a molecular weight of approximately 150,000 kDa, binds Dectin-1 with an IC$_{50}$ of 2.7pM (Figure 7A) (manuscript in preparation). Laminarin, a soluble glucan derived from \textit{Laminaria digitata} with a molecular weight of approximately 7500 kDa, binds Dectin-1 with an affinity of almost 100 times less that of GP (Figure 7A, manuscript in preparation). The \textit{in vitro} effects of these different glucans on Dectin-1 have also been found to be different (Herre et al. 2004). GP induces internalization and degradation of Dectin-1 after binding, while laminarin induces internalization, but the receptor recycles quickly back to the cell surface (Herre et al. 2004). Finally, \textit{in vivo} laminarin is not protective in polymicrobial sepsis (Figure 7B), though GP is. The molecular basis of the differences in activity between different glucans
Intracellular Trafficking of PRR/PAMP Complexes

As previously mentioned, PRRs mediate internalization of PAMPs. The complex is then trafficked within the cell. Ideally, the microbe is destroyed within a phagolysosome, and the receptor is either transported back to the cell surface or is degraded. The exact molecular mechanisms for internalization and trafficking vary by PRR and may vary by which PAMP is bound. Additionally, the signal generated by the PRR/PAMP interaction may be dependent on trafficking. There are two primary mechanisms for receptor/ligand internalization: clathrin-mediated and clathrin independent lipid raft-mediated (Le Roy and Wrana, 2005).

Figure 7 Different glucans have varying affinities for Dectin-1 and differing \textit{in vivo} activities. A. IC$_{50}$ for each glucan was determined by surface plasmon resonance. The binding curves for glucan phosphate and laminarin demonstrate a higher Dectin-1 binding affinity for GP than for laminarin. B. The survival curve for CLP induced polymicrobial sepsis in the presence and absence of laminarin treatment reveals that laminarin is not protective in sepsis. Manuscript in preparation.

\textit{in vivo} is not yet known, but the differences in binding affinity to Dectin-1 and effects on Dectin-1 trafficking are likely involved.
**Clathrin Mediated Internalization.** The internalization of many nutrients, pathogens, growth factors, and receptors is clathrin dependent (Le Roy and Wrana, 2005). Clathrin is a cytoplasmic protein that interacts with ligand-bound receptor via adapter proteins to form a coat along the plasma membrane (Le Roy and Wrana, 2005). The clathrin coated pit then mediates internalization of the plasma membrane and the receptor/ligand complex (Figure 8A) (Le Roy and Wrana, 2005). Upon internalization, the resulting vesicle is uncoated and fuses with early endosomes (Figure 8A) (Le Roy and Wrana, 2005). The fate of the receptor/ligand complex then varies. The receptor may dissociate from the ligand, usually in late endosomes (Figure 8A) (Le Roy and Wrana, 2005). The receptor then may recycle via recycling endosomes to the cell surface or be degraded (Figure 8A) (Le Roy and Wrana, 2005). Alternately, the receptor/ligand complex may be trafficked to lysosomes where it and the ligand are degraded (Figure 8A) (Le Roy and Wrana, 2005). Studies have also found that the complex may be trafficked to the Golgi apparatus (Figure 8A) (Le Roy and Wrana, 2005). Therefore, the fate of the receptor/ligand complex after clathrin mediated internalization varies widely depending on the receptor and ligand involved.

**Non-clathrin Dependent Internalization.** Evidence suggests that clathrin independent internalization is mediated by lipid rafts (Nichols 2003; Le Roy and Wrana, 2005). Lipid rafts are cholesterol rich plasma membrane micro-domains (Nichols 2003; Le Roy and Wrana, 2005). They are able to segregate membrane components and are thought to be platforms for signal transduction by cell surface receptors (Le Roy and Wrana, 2005). Caveolae are one type of lipid raft micro-domain (Williams and Lisanti 2004c). They are membrane invaginations that are dependent on caveolin for their formation (Le Roy and Wrana, 2005). Lipid raft mediated
internalization may be mediated by caveolin, but caveolin independent mechanisms also occur (Le Roy and Wrana, 2005). Upon internalization by caveolin the receptor/ligand complex is trafficked in a vesicle known as a caveosome (Figure 8B) (Le and Nabi, 2003; Nichols 2003; Le Roy and Wrana, 2005). The caveosome traffics to the endosomal pathway as well as to the Golgi apparatus and the endoplasmic reticulum (Figure 8B) (Le and Nabi, 2003; Le Roy and Wrana, 2005). Non-caveolin dependent lipid raft mediated internalization is poorly understood at this time.

Figure 8  Schematic representation of the 2 types of receptor mediated internalization, clathrin mediated and lipid raft dependent
**Internalization and Trafficking of PRR/PAMP Complexes.** The mechanisms of intracellular trafficking have primarily been investigated for receptors that interact with endogenous ligands such as growth factor and cytokine receptors; however, the means of internalization and trafficking of PRRs are slowly coming to light. The majority of studies have investigated the trafficking pathways of the TLRs, specifically TLR2 and TLR4 (Thieblemont and Wright 1999; Triantafilou et al. 2002; Triantafilou et al. 2004a; Triantafilou et al. 2004b). For instance, when TLR2 interacts with lipoteichoic acid, the complex is internalized by a lipid raft dependent mechanism and is then trafficked to the Golgi apparatus (Triantafilou et al. 2004a). Trafficking of LPS by TLR4 is a bit more complex (Thieblemont and Wright 1999; Triantafilou et al. 2002; Triantafilou et al. 2004a; Triantafilou et al. 2004b). TLR4 has been found to migrate to lipid rafts upon interacting with LPS (Triantafilou et al. 2002; Triantafilou et al. 2004b); however, internalization of the complex is inhibited by loss of clathrin and is retained with disruption of the lipid raft (Husebye et al. 2006). This indicates that TLR4/LPS uptake is mediated by clathrin. The TLR4/LPS complex then localizes to the early endosomes (Husebye et al. 2006). At later time intervals the receptor and ligand dissociate, with the LPS being found in the Golgi apparatus, while TLR4 is transported to the lysosome where it is degraded (Thieblemont and Wright 1999; Latz et al. 2002; Husebye et al. 2006). The dependency of signaling on trafficking is disputed (Latz et al. 2002; Cuschieri 2004). Disruption of the Golgi apparatus had no effect on LPS induced NF-κB activation but blocked LPS induced IL-8 production (Latz et al. 2002). However, disruption of lipid rafts attenuated LPS induced production of TNFα but increased production of IL-10 leading to an anti-inflammatory phenotype (Cuschieri 2004).

The knowledge of intracellular trafficking of glucan and Dectin-1 is limited. For many years it has been assumed that glucans were internalized into endosomes and transported to
lysosomes where they were degraded. These studies were performed prior to the discovery of lipid raft mediated internalization and were done using electron microscopy. The glucan containing vesicles were assumed to be endosomes and lysosomes based upon their structure. More recent studies have found that upon binding glucan, the Dectin-1/glucan is rapidly internalized and trafficked to an unknown organelle (Herre et al. 2004). Additionally, the fate of the Dectin-1 was dependent on the activity of the glucan (Herre et al. 2004). Binding of the biologically inactive glucan laminarin resulted in recycling of Dectin-1 to the cell surface, while binding of GP, which is known to have immunomodulatory properties, resulted in Dectin-1 degradation (Herre et al. 2004). Studies by McCann et al. found that particulate glucans are internalized by a non-caveolin dependent mechanism into endosomes and are trafficked to lysosomes (McCann et al. 2005). This study also demonstrated that cell stimulation was not dependent on glucan internalization (McCann et al. 2005). These authors did not determine the means of internalization, nor did they investigate trafficking of soluble glucans. Furthermore, the use of fluorescence microscopy rather than confocal microscopy diminishes confidence in these data. Finally, studies by Karinaga et al. demonstrated that PEG-appended schizophyllan could be used to deliver oligonucleotides into cells and avoid lysosomal degradation (Karinaga et al. 2005). Therefore, a more complete study is required to address trafficking by the Dectin-1/glucan complex.
Chemical Mediators of the Immune Response

Cytokines. Cytokines are soluble chemical mediators of the immune response. They are produced by both classic immune cells and cells that are not considered to be part of the immune system. There are hundreds of different cytokines, and most cytokines had a myriad of different effects. For clarification cytokines can be sub-divided into different types based upon their activities. The pro-inflammatory cytokines tend to stimulate the immune response by activating immune cells. This group includes TNFα, IL-1β, and IL-2 (http://www.copewithcytokines.de). Anti-inflammatory cytokines tend to blunt the immune response by inhibiting the production of pro-inflammatory cytokines and preventing activation of immune cells. This group includes IL-10 as well as the IL-1 receptor antagonist (ra) and the soluble receptor for TNFα (http://www.copewithcytokines.de). Cytokines may also be grouped based upon the type of adaptive immune response they induce. The Th1 cytokines stimulate a cell mediated immune response and are represented by interferon (IFN) γ (http://www.copewithcytokines.de). IL-12 and IL-18, which stimulate the production of IFNγ, are also considered to be in this group (http://www.copewithcytokines.de). The Th2 cytokines stimulate the humoral or antibody mediated immune response. These cytokines also inhibit the cell mediated immune response and include IL-4 and IL-5 (http://www.copewithcytokines.de). IL-6 is an important cytokine that is not easily grouped. It is primarily an inflammatory cytokine as it activates macrophages and neutrophils, but it also stimulates the production of anti-inflammatory mediators (http://www.copewithcytokines.de). Immune cell growth factors are also considered cytokines. These mediators stimulate the production of immune cells in the bone marrow. This group includes GM-CSF and G-CSF (http://www.copewithcytokines.de).
**Chemokines.** Chemokines are molecules that stimulate cells to migrate to areas of inflammation. Many of these molecules also act as cytokines in that they will activate the cells that they attract. Chemokines have been discovered for lymphocytes, monocytes, and neutrophils. Many of these chemokines will attract multiple cell types. IP-10 and monokine induced by IFN (MIG) attract T lymphocytes (http://www.copewithcytokines.de). Monocyte chemoattractant protein (MCP) and macrophage inflammatory protein (MIP) 1α primarily attract monocytes and macrophages (http://www.copewithcytokines.de). IL-8, IL-17, and keratinocyte-derived chemokine (KC) attract and activate neutrophils (http://www.copewithcytokines.de).

**Critical Illness**

*C. albicans*

*C. albicans* is a dimorphic fungus that is part of the normal flora of the oropharynx and GI tract (Calderone and Fonzi 2001). *C. albicans* can cause a wide range of infections from superficial mucosal infections in individuals with normal immune function to life threatening systemic infections in the critically ill, immunocompromised host (Calderone and Fonzi 2001; Sullivan et al. 2004). *C. albicans* is the most frequent fungal pathogen in critically ill and/or immunocompromised patients (Wilson et al. 2002; Sullivan et al. 2004). The mortality rate for disseminated candidiasis is greater than 35% and has remained steady despite the discovery of multiple anti-fungal drugs (Sullivan et al. 2004). We do not fully understand the cellular and molecular changes that occur in the immunocompromised and/or critically ill patient that render them susceptible to life threatening fungal infections, but disseminated or systemic *C. albicans* infection is often associated with neutropenia and/or dysfunctional cell mediated immunity (Bodey 2000).
**C. albicans and the Innate Immune Response.** Despite the medical significance of *C. albicans*, there is still much that we do not know about the cellular and molecular mechanisms by which *C. albicans* species and other fungal pathogens interact with and are recognized by the innate immune system. The *C. albicans* cell wall contains several PAMPs that may be recognized by PRRs in the innate immune system, i.e. mannan, phospholipomannan, and glucan (Chauhan et al. 2002; Roeder et al. 2004). PRRs, such as Toll Like Receptor 2 (TLR2) (Jouault et al. 2003; Roeder et al. 2004), TLR4 (Tada et al. 2002; Roeder et al. 2004), DC-SIGN (Cambi et al. 2003) the Type 3 complement receptor (CR3) (Forsyth et al. 1998) and the mannose receptor (Allavena et al. 2004), have been reported to be involved in recognition and response to these fungal cell wall PAMPs, but the role of these receptors in the response to *C. albicans* infection has been disputed (Bellocchio et al. 2004; Netea et al. 2004). For example, TLR2, which binds phospholipomannan (Jouault et al. 2003; Roeder et al. 2004) and is involved in the response to glucan (Gantner et al. 2003), was thought to be essential for protection against fungal infection (Villamon et al. 2004). However, recent studies have shown that TLR2 does not protect mice from disseminated candidiasis (Bellocchio et al. 2004; Netea et al. 2004). Indeed, genetic depletion of TLR2 improves survival outcome in candidiasis (Bellocchio et al. 2004; Netea et al. 2004). Additionally, Lee et al. have reported that mannose receptor knock-out mice were no more susceptible to disseminated candidiasis than wild type (Lee et al. 2003). Thus, the question of which receptor(s) in the innate immune system are necessary for defense against *C. albicans* remains unanswered.

Neutrophils are integral to the innate immune response to *C. albicans* infection (Kullberg et al. 1999), and cytokines and chemokines are necessary to activate and attract neutrophils to
areas of infection. Mice infected with *C. albicans* have increased circulating levels of TNFα, IL-6, and IL-17 (Steinshamn and Waage 1992; Huang et al. 2004). Studies using knock-out mice have revealed that loss of TNFα or IL-6 significantly increases susceptibility of mice to *C. albicans* infection (Romani et al. 1996; Mencacci et al. 1998). Additionally, IL-17 knock-out mice infected with *C. albicans* have a reduced survival rate when compared to wild type controls (Huang et al. 2004). In the case of both IL-6 and IL-17 depletion, animals had impaired neutrophil recruitment and activity (Romani et al. 1996; Huang et al. 2004). The adaptive cell mediated immune response is also necessary to eliminate fungal pathogens. IFNγ, which stimulates a cell mediated adaptive immune response, is thought to play an important role in innate host defense against *C. albicans* infection, as genetic depletion of IFNγ reduces survival in mice infected with *C. albicans* (Balish et al. 1998). Cytokines that stimulate the production of IFNγ, such as IL-12 and IL-18, are also integral to an effective cell mediated immune response to *C. albicans* (Netea et al. 2003; Bjorkbacka et al. 2004). These studies confirm the complexity of an effective immune response against *C. albicans*.

*C. albicans*, Glucan, and Dectin-1. Dectin-1 is thought to be an important sentinel receptor for fungal infections, especially *C. albicans* (Brown et al. 2003). Dectin-1 has been found to bind to *C. albicans* blastospore cells both as a soluble recombinant protein or expressed on the surface of transfected cells (Brown et al. 2003; Gantner et al. 2005). The interaction of *C. albicans* with cellular Dectin-1 results in internalization of the yeast and production of TNFα and reactive oxygen species (Brown et al. 2003; Gantner et al. 2005). While these *in vitro* studies have demonstrated the relationship between Dectin-1 and *C. albicans*, there are very little
data available on the in vivo role of Dectin-1 in fungal infection. Studies have shown systemic administration of GP increases resistance to *C. albicans* infection (Williams et al. 1978; Williams et al. 1991b). Specifically, glucan treated mice show decreased renal pathology and increased survival outcome in response to *C. albicans* infection (Williams et al. 1991b). These data indicate that Dectin-1 is involved in the immune response to *C. albicans*, but the effect of *C. albicans* on Dectin-1 in vivo is unknown.

**Polymicrobial Sepsis**

The critically ill patient frequently develops a complex disease spectrum that may include systemic inflammatory response syndrome (SIRS), sepsis syndrome and/or septic shock (Oberholzer et al. 2001). These conditions are characterized by fever, tachycardia, tachypnea, and shock (Brunn and Platt 2006). Sepsis generally refers to the patient with an infectious etiology, while those with SIRS have no demonstrable infection (Brunn and Platt 2006). In the United States ~750,000 patients/year develop sepsis syndrome (Angus et al. 2001). Of these patients, 51.1% receive intensive care and the overall mortality rate is 28.6% (~215,000 deaths/year) (Angus et al. 2001). The annual cost of sepsis syndrome is ~$16 billion dollars/year (Angus et al. 2001). At present, we do not understand the cellular and molecular mechanisms that are involved in the initiation and propagation of septic injury; nor do we understand the physiologic mechanisms that attempt to maintain homeostasis and promote survival in the septic patient. In addition, attempts at developing effective therapies for sepsis syndrome have proven to be exceedingly difficult. Consequently, survival outcome in sepsis syndrome has not significantly improved over the last few decades (Oberholzer et al. 2001).
Sepsis, SIRS, and the Innate Immune Response. Severe injury or infectious challenge results in the release of multiple exogenous (PAMPs) and endogenous ligands for PRRs present on immune cells (Brunn and Platt 2006). The interaction of PRRs with their ligands results in the release of pro-inflammatory mediators. The most studied of the PRRs involved in the etiology of sepsis and SIRS is TLR4. LPS, the exogenous ligand for TLR4, is released into the circulation by Gram negative bacteria. Administration of LPS in the absence of infection has been used as an experimental model of septic shock (Lowry 2005). TLR4 also has several endogenous ligands including the extra-cellular matrix protein heparan sulfate (Johnson et al. 2002). These ligands are be released into the systemic circulation by proteases activated by trauma or local inflammation (Johnson et al. 2002; Brunn and Platt 2006). Localized release of these ligands results in a localized immune response, but if the amount of ligand is great enough, it may be released into the systemic circulation. The innate immune system may respond by over-expressing inflammatory mediators resulting in a systemic inflammatory response that culminates in severe shock, multi-organ failure, and death (Oberholzer et al. 2001).

Cytokines are believed to be intimately involved in the pathogenesis of sepsis, though their exact role is not known. It has been demonstrated that sepsis increases the circulating levels of the inflammatory cytokines TNFα and IL-6, and sustained elevations of these cytokines correlates with severity of illness and mortality (Damas et al. 1992; Pinsky et al. 1993). Additionally, administration of TNFα or IL-1 to rodents and rabbits yields a condition that mimics sepsis (Tracey et al. 1986; Bauss et al. 1987; Okusawa et al. 1988). These studies suggest that the inflammatory cytokines are the primary mediators of sepsis; however, inhibition of TNFα and IL-1 was found to be protective only in animal models of sepsis while having no effect in human patients (Beutler et al. 1985; Ohlsson et al. 1990; Opal et al. 1997; Clark et al.
Further complicating the issue is the role of anti-inflammatory cytokines and soluble inflammatory cytokine inhibitors. Elevated levels of IL-10, IL-1ra, and the soluble receptor for TNFα have been found in patients with severe sepsis (Marchant et al. 1994; Neidhardt et al. 1997; Gogos et al. 2000). This elevation contributes to the compensatory anti-inflammatory response syndrome thought to follow the SIRS in cases of severe sepsis (Bone et al. 1997). How the interaction of pro-inflammatory and anti-inflammatory cytokines relates to sepsis and survival in sepsis is not understood.

Sepsis, Glucan, and Dectin-1. As previously mentioned, administration of GP improves survival rates in CLP induced polymicrobial sepsis. The molecular mechanisms responsible for protection are not fully known, but glucan ligands blunt the early increase in tissue transcription factor activation, thereby limiting the host pro-inflammatory response to the septic injury (Williams et al. 1999b). Preventing early activation of nuclear factor (NF)-κB and NF-IL6 positively correlated with improved long-term survival (Williams et al. 1999b). Additionally, glucan administration stimulated the phosphoinositide 3 kinase (PI3K) signaling pathway, which correlated with improved outcome in CLP sepsis (Williams et al. 2004a). PI3K has been shown to mediate a diverse array of effects and has been speculated to be a negative feedback mechanism for innate immune responses (Guha and Mackman 2002; Fukao and Koyasu 2003). Blockade of the PI3K pathway with wortmannin completely eliminated the protective effect of glucan in sepsis, thus demonstrating that the effect is mediated through a PI3K dependent mechanism (Figure 9) (Williams et al. 2004a). As the induction of a response to glucan begins with binding of glucan to PRRs on the cell surface, it is highly likely that Dectin-1 is involved in the pathogenesis of sepsis. Additionally, septic patients have elevated levels of circulating...
Figure 9  Inhibition of PI3K increases abrogates the protection induced by glucan phosphate administration mice polymicrobial sepsis. Administration of the PI3K inhibitor wortmannin increases mortality in CLP (A). Glucan induced protection in CLP is lost with wortmannin treatment (B) (Adapted from Williams et al. 2004a).

glucans (Digby et al. 2003). How Dectin-1 may be affected by circulating glucans in sepsis or by administration of glucans in sepsis has yet to be determined.
CHAPTER 2

EXPERIMENTAL APPROACH

An appropriate innate immune response is integral to survival in trauma and infection. Binding of PAMPs to PRRs and the cell signaling resulting from this interaction are the first steps in the generation of the innate immune response. Though much has been discovered regarding the signaling pathways resulting from the interaction of some PRRs with their PAMPs, a complete understanding of how these pathways generate an appropriate immune response has not been achieved. Additionally, the mechanisms that result in an inappropriate response have not been determined. The reasons for this lack of understanding are because of the extreme complexity of the innate immune system. Over time more cell types have been found to be involved in innate immunity, and additional PRRs continue to be discovered. Additional signaling pathways used by PRRs also continue to be elucidated, as do the ligands bound by PRRs. Finally, the effects of the innate immune response on the adaptive immune response are only now coming to light.

The majority of information about PRRs has been discovered studying the TLR family of receptors. However, other PRRs do exist and may be as important as the TLRs with regards to innate immunity. Dectin-1 is a newly discovered PRR. Studies have shown that Dectin-1 is a major PRR for the fungal PAMP, glucan (Brown and Gordon 2001; Willment et al. 2001; Brown et al. 2002; Taylor et al. 2002; Brown et al. 2003). As it mediates an inflammatory response when bound to glucan as a single molecule or present in the fungal cell wall, Dectin-1 has been proposed to be a sentinel receptor for fungal infection (Brown et al. 2003). However, the studies investigating the interaction between Dectin-1 and fungi have been performed in vitro; therefore, little is known about the effect of C. albicans infection on Dectin-1 in vivo. As the ligand for Dectin-1 improves survival in polymicrobial sepsis, it is possible that Dectin-1 also plays a role
in the pathophysiology of polymicrobial sepsis (Williams et al. 1999b; Williams et al. 2004a). Though previous studies have determined that the Dectin-1 ligand GP is blunting the pro-inflammatory NF-κB pathway and stimulating the anti-apoptotic PI3K pathway in polymicrobial sepsis, the role of Dectin-1 in these activities is has not been discovered (Williams et al. 1999b; Thomas et al. 2003). Additionally, elevated levels of circulating glucans have been found in the critically ill, regardless of the presence of fungal or bacterial infection (Digby et al. 2003). It is likely that these glucans are interacting with Dectin-1 on innate immune cells. The consequences of this interaction are unknown. *In vivo* studies investigating the effects of fungal and polymicrobial sepses on Dectin-1 are necessary to begin to address the role of Dectin-1 in critical illness.

Little is known about the effects of the administration of glucan on Dectin-1 *in vivo*, as the majority of studies investigating this interaction have also been performed *in vitro* (Brown et al. 2002; Taylor et al. 2002; Brown et al. 2003; Gantner et al. 2003; Gantner et al. 2005; Underhill et al. 2005). The *in vitro* studies have used single cell conditions, and frequently the cells used are transfected cell lines. It is possible that these results will not translate in the much more complex *in vivo* milieu. Additionally, these studies were performed primarily using the glucan-containing particle, zymosan (Brown et al. 2002; Taylor et al. 2002; Brown et al. 2003; Gantner et al. 2003). Zymosan is a product of the *S. cerevisiae* cell wall. It contains a large amount of glucan but is contaminated with other cell wall molecules including mannan. Therefore, the effects seen with zymosan are not necessarily attributable to glucan alone. This is supported by the fact that pure glucans such as GP do not have the same effects as zymosan and, in many cases, have been used to block the effects of zymosan (Brown et al. 2003). It has even been suggested that GP is actually a Dectin-1 antagonist; however, this is unlikely as GP is a potent immunomodulator *in vivo*. Studies regarding the *in vivo* effects of GP on Dectin-1 will clarify this issue.
Dectin-1 is known to mediate the internalization of glucan (Brown et al. 2002; Herre et al. 2004). The mechanism for this internalization has not been determined, though blockade of caveolin-1 does not inhibit glucan uptake (McCann et al. 2005). Furthermore, trafficking of the Dectin-1/glucan complex is poorly understood and may relate to \textit{in vivo} glucan activity (Herre et al. 2004). Glucans that have biologic activity result in degradation of Dectin-1 upon internalization of the Dectin-1/glucan complex, and the receptors remain lost from the surface until new receptors have been synthesized (Herre et al. 2004). Though Dectin-1 mediated signaling has been reported to be independent of internalization, it is possible that differences in trafficking are responsible for differences in biologic activity (Brown et al. 2003; Herre et al. 2004; McCann et al. 2005). Therefore definitive studies are needed to elucidate the mechanisms for internalization of the Dectin-1/glucan complex, and to determine how glucans are trafficked upon internalization.

Based upon previous studies regarding Dectin-1 and glucans, we have formed the following hypotheses.

1. Fungal sepsis will alter Dectin-1 expression \textit{in vivo}.
2. Polymicrobial sepsis will alter Dectin-1 expression \textit{in vivo}.
3. Biologically active glucans will alter Dectin-1 expression \textit{in vivo}, while inactive glucans will not.
4. Biologically active glucans will be trafficked differently than inactive glucans.

These hypotheses will be tested in the following 4 specific aims.

Specific Aim 1: Determine the effect of systemic fungal infection on Dectin-1 \textit{in vivo}.
Specific Aim 2: Determine the effect of polymicrobial sepsis on Dectin-1 \textit{in vivo}.
Specific Aim 3: Determine the effect of the administration of glucans, active and inactive, on Dectin-1 \textit{in vivo}.
Specific Aim 4: Determine how glucans, active and inactive, are trafficked within the cell.

Experimental Design

Specific Aim 1. Determine the effect of systemic fungal infection on Dectin-1 in vivo.
Mice will be injected with *C. albicans*, a clinically relevant fungal pathogen. Mice injected with an equal volume of diluent will be used as controls. One group will be followed for survival. The remaining mice will be harvested at appropriate times throughout the course of infection. Dectin-1 expression and cell type will be determined on blood and splenic leukocytes by flow cytometry. Dectin-1 expression will be determined in the kidney by immunohistochemistry. Blood, kidney, and lung will be cultured for *C. albicans*. Circulating cytokine and chemokine concentrations will be determined by Luminex.

Specific Aim 2. Determine the effect of polymicrobial sepsis on Dectin-1 in vivo.
Mice will undergo CLP to induce polymicrobial sepsis. No surgery mice will act as normal control, and mice that have had laparotomy only will serve as anesthesia and surgery controls. Half of each group will receive GP (1 mg/mouse) 1 h prior to surgery. One group will be followed for survival. The remaining mice will be harvested at appropriate time intervals throughout the course of infection. Dectin-1 expression and cell type will be determined on blood, peritoneal, and splenic leukocytes by flow cytometry. Circulating cytokine concentrations will be determined by Luminex.

Specific Aim 3. Determine the effect of the administration of glucans, active and inactive, on Dectin-1 in vivo.
Mice will be injected with 1 mg/mouse GP, laminarin, mannan, or pullulan. Control animals will receive an equal volume of diluent. Mice will be harvested at regular intervals over the course of 10 d. Dectin-1 expression and cell type will be determined on blood, peritoneal, and splenic leukocytes by flow cytometry. Circulating cytokine and chemokine concentrations will be determined by Luminex. A subset of mice will receive 5 mg/mouse fluorescent labeled GP. Blood and peritoneal leukocytes will be evaluated by confocal microscopy and flow cytometry for GP uptake.

**Specific Aim 4.** Determine how glucans, active and inactive, are trafficked within the cell.

Macrophages will be harvested from mouse peritoneal cavities after stimulation with thioglycollate. Cells will be treated with fluorescent labeled GP, laminarin, or particulate glucan. A subset of cells will be treated with clathrin or lipid raft internalization inhibitors. Cells cultured in 6 well plates will be evaluated for Dectin-1 and glucan internalization by flow cytometry after 3 h incubation with glucan. Cells cultured on 4 chamber slides will be fixed and permeablized after varying incubation times with glucan. The cells will be stained with fluorescent labeled antibodies against caveolin-1, clathrin, rab5, GM130, lamp1, GRP78, Dectin-1, the p85 subunit of PI3K, SR-A, or TLR2. The cells will be imaged by confocal microscopy and analyzed for glucan co-localization.
Animals

Age- and weight-matched adult male ICR/HSD mice were obtained from Harlan Sprague Dawley (Indianapolis, IN). Caveolin-1 deficient mice (Cav-/-) and their wild type (WT) control (B6129SF2/J) were obtained from The Jackson Laboratory (Bar Harbor, ME). The animals were maintained on standard laboratory chow and water ad libitum with a 12-hour light/dark cycle. Serologic testing confirmed that the mice were virus free prior to study entry. All animal procedures were reviewed and approved by the University Committee on Animal Care at the James H. Quillen College of Medicine, East Tennessee State University.

Cell Culture

Elicited macrophages were harvested by peritoneal lavage 72 h after IP injection of thioglycollate (2 ml). Elicited neutrophils were harvested by peritoneal lavage 18 h after IP injection of thioglycollate. Both cell types were maintained in RPMI-1640 medium (Sigma, St Louis, MO) supplemented with 10% fetal calf serum (FCS), penicillin, and streptomycin. Macrophages were allowed to adhere overnight prior to treatment, and neutrophils were treated just after harvest.
**Candida albicans**

A laboratory strain of *C. albicans* was used that produced true germ tubes within 2 h when incubated at 37°C in FCS. The strain is a human vaginal isolate that has been used as a laboratory control strain since the early 1980s. No information on the effects of this strain was available in mice. A blood agar plate (BAP) was streaked with a control culture of *C. albicans* and incubated at 37°C for 48 h. A 5 ml tube of Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, MI) was inoculated with 1 colony of *C. albicans* and incubated at 37°C in a slanted position with agitation at 200 rpm for 18 h for production of a maximal biomass of blastospores. The blastospores were washed 3 times with sterile physiologic saline by centrifugation and suspended to 5 ml. A viable count was determined by serial 10-fold dilutions in saline and triplicate plating of 1 µl of each dilution on BAP. The stock was suspended in 5% dextrose in water to produce a final concentration of 5x10⁵ cfu/ml.

**Carbohydrates**

Water soluble *Saccharomyces cerevisiae* derived glucan phosphate (GP) was prepared and chemically characterized as previously described (Williams et al. 1991a; Ensley et al. 1994; Mueller et al. 1994; Mueller et al. 1995; Lowman et al. 1998). The final product was stored (-80°C) as a lyophilized powder. It was dissolved in dextrose 5% w/v (D5W, Baxter, Toronto, Canada), filter sterilized (0.45 µm), and screened for endotoxin contamination with the Endospecy assay (Seikagaku, Tokyo), which is specific for endotoxin but does not respond to (1→3)-β-D-glucans (Kambayashi et al. 1991; Miyazaki et al. 1995). Laminarin (Sigma, St. Louis, MO), a biologically inactive (1→3)-β-D-glucan, was used to further investigate the role of Dectin-1. Mannan (Sigma, St. Louis, MO) and pullulan (Pfanstiehl, Waukegan, IL) were used
as control carbohydrates as they are known to not interact with Dectin-1. The carbohydrates were dissolved in D5W, incubated overnight in pre-washed polymyxin coated agarose beads (BioRad, Hercules, CA) to remove endotoxin, and filter sterilized prior to use. Diaminopropane derivatized GP, laminarin, and particulate glucan (Kougias et al. 2001) were labeled with Alexa Fluor 488 (AF488) or AF647 (Molecular Probes, Eugene, OR) by hydrating the glucan in 0.1 M borate buffer, pH 8.5, dissolving the succidamidyl ester form of AF488 or AF647 in DMSO to a concentration of 100ug/ml, and incubating the 2 in the dark overnight with rotation at ambient temperature. Excess dye was removed by dialysis against water until dye was no longer visible in the dialysate. The labeled product was then buffer exchanged into D5W. Conjugation and binding activity were confirmed by incubation of the labeled glucan with Dectin-1 transfected cells for 30m at 4ºC, washing 3 times with phosphate buffered saline (PBS) and analyzing by flow cytometry.

**Polymicrobial Sepsis**

Cecal ligation and puncture was performed to induce polymicrobial sepsis (Baker et al. 1983; Williams et al. 1999a; Yang et al. 2002;). Briefly, mice were anesthetized with isoflurane, and a ventral midline incision was made. The cecum was externalized and the cecal contents were milked to the distal end of the cecum. The cecum was ligated with 0 silk suture distal to the ileocecal junction and two punctures were made in non-vascular areas with an 18g needle. A small amount of ingesta was extruded from the punctures, the cecum was returned to the abdominal cavity, and the wound was closed in 2 layers. The mice were recovered with 1 ml lactated ringers solution subcutaneously. Sham surgery (laparotomy alone) mice were used as a control for surgery and anesthesia, and animals that underwent no surgery or anesthesia were used as negative controls.
In Vivo Experimental Protocols

C. albicans Infection. Mice were administered $1 \times 10^5$ C. albicans IV. Control mice received diluent IV. Spleens and blood were harvested at 1, 3, 5, and 7 days post-injection for flow analysis. Kidney, lung, and blood were also harvested for culture, and serum was harvested for cytokine concentration. Kidneys were harvested for immunohistochemistry. A parallel group of mice were administered $1 \times 10^5$ C. albicans cells IV and survival was followed for up to 21 days.

Polymicrobial Sepsis. One hour prior to surgery, mice were administered 1 mg GP or an equal volume of 5% Dextrose in water IP. The treatment groups were: i) no surgery (control), ii) sham surgery (laparotomy only), iii) cecal ligation and puncture (CLP), iv) glucan without surgery, v) sham surgery + glucan, vi) CLP + glucan. Mice were sacrificed at 0, 3, 6, 12, and 24h post-operatively and peritoneal cells, splenocytes and peripheral blood leukocytes were obtained for flow cytometric analysis. Serum was harvested at 12 h post-operatively for cytokine analysis. Parallel groups of untreated and glucan treated CLP mice were monitored for survival for 14 d.

Glucan Injection. At time 0 mice were given either 1 mg GP, 1 or 5 mg AF488-GP, 1 mg laminarin, 1 mg mannann, 1 mg pullulan, or an equal volume of D5W intravenously (IV) or intraperitoneally (IP). Mice were sacrificed at 0 h, 3 h, 1, 3, 5, 7, and 10 d post-injection and peritoneal cells, bone marrow cells, splenocytes, and peripheral blood leukocytes were obtained for flow cytometry or confocal microscopy. Serum was collected at 0, 3, 24, and 72 h post-IV injection for cytokine analysis.
**In Vitro Experimental Protocols**

**Dectin-1 Internalization Studies.** Thioglycollate elicited neutrophils were cultured in 12 well plates (Corning Incorporated, Corning, NY) at 500,000 cells/well. The cells were incubated with 1 µg/ml S. cerevisiae derived GP, C. albicans blastospore derived GP, C. albicans hyphal derived GP, S. cerevisiae derived mannan (Sigma), or E. coli O55:B5 LPS (Sigma) alone or in combination for 3 h.

**Internalization Inhibitors.** Thioglycollate elicited macrophages from ICR/HSD mice, Cav-/- mice or WT mice were cultured in 6 well plates (Corning Incorporated) at 750,000 cells/well and allowed to adhere overnight. The cells were incubated with 10 µg/ml fluorescent labeled GP for 3 h. Cells were incubated with either media alone, 5 mM methyl β cyclodextran (MβCD, Sigma) to disrupt lipid rafts, or 500 mM sucrose (Fisher Scientific, Fair Lawn, NJ) to inhibit clathrin polymerization for 1 h prior to addition of the GP.

**Flow Cytometry**

Peritoneal cells were obtained by peritoneal lavage. Bone marrow cells were obtained by flushing the femoral marrow cavities with sterile PBS. Splenocytes were isolated by teasing apart the spleens and separation of the splenic stroma by sedimentation. Whole blood was collected into EDTA Microtainer tubes (Becton Dickenson Vacutainer Systems, Franklin Lakes, NJ). Red blood cells were lysed in all samples using PharmLyse buffer (Pharmingen) according to the manufacturer’s directions. Cultured macrophages were scraped into Pharmingen stain buffer, and non-adherent cultured neutrophils were harvested and then washed with stain buffer. Cells were blocked with 5% rabbit serum, 0.5% bovine serum albumin, and 5 mM EDTA with anti-murine CD16/32 (Pharmingen) prior to staining. Staining was performed according to Becton Dickenson protocols at 4°C. Cells were stained with biotin conjugated rat anti-murine
Dectin-1 (2a11) (Brown et al. 2003), biotin conjugated goat anti-murine Dectin-1 (R and D Systems, Minneapolis, MN), Fluorescein isothiocyanate conjugated anti-neutrophil (clone 7/4) (Serotec Oxford, UK), Allophycocyanin conjugated anti-F4/80 (Caltag, Burlingame, CA), and Peridinin chlorophyll-A protein conjugated anti-CD3 (Pharmingen, San Diego, CA) or their isotype control antibodies. Biotinylated antibodies were detected by streptavidin-phycoerythrin (Pharmingen). Cells were suspended in Pharmingen Stain Buffer and analyzed using a FACScalibur flow cytometer with CellQuest software (BD Biosciences, Mountain View, CA).

Cytokine and Chemokine Analysis

Blood was collected into serum separator Microtainer tubes (Becton Dickenson Vacutainer Systems), spun at 2000 rpm, and the serum was harvested and stored at -80°C until analysis. Serum samples were diluted 1:2 with PBS prior to assay. The assay was performed using the Biosource Mouse Multiplex 10 cytokine kit (Camarillo, CA) that includes tumor necrosis factor alpha (TNFα), interleukin (IL)-1 beta, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, interferon gamma (IFNγ), and granulocyte monocyte colony stimulating factor (GM-CSF) with an additional 6 chemokines (cytokine-induced neutrophil chemoattractant (KC), interleukin (IL)-17, inducible protein (IP)-10, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, and monokine induced by gamma interferon (MIG)) according to manufacturer’s instructions. The samples were analyzed on a Luminex 100 (Austin, TX). Cytokine concentrations were established with MasterPlex QT software v. 1.2.8.58 (MiraiBio, Inc. Alameda, CA).
Blood and Tissue Culture of \textit{C. albicans}

Spleen, kidney, lung, and blood were collected by aseptic surgical procedures. Blood (250µl) was incubated 1 week in brain heart infusion broth at 30ºC. The organs were dipped into 70% ETOH and flamed over a Bunsen burner. They were placed individually into a sterile tissue grinder and homogenized into 1 ml saline. Two drops from a sterile Pasteur pipette (~50µl) of the organ mixture or the blood culture were placed on duplicate blood agar plates and streaked for isolation. After incubation at 30ºC for 48h, the amount of growth was determined by counting the largest number of quadrants that contained colonies of \textit{C. albicans}. The identification of \textit{C. albicans} was established by Gram stain and germ tube formation in FCS at 37ºC for 2 h.

Histopathology

Tissues were collected into 10% buffered neutral formalin (Fisher Scientific, Fair Lawn, NJ), paraffin embedded and then sliced into 5 micron sections. The sections were stained with hematoxylin and eosin (H&E) or fungal periodic acid Schiff (PAS) reagent.

Immunohistochemistry

Tissues were collected, embedded in “Tissue-Tek” OCT compound (Miles Inc., Elkhart, IL), and snap frozen with isopentane and liquid nitrogen. The tissues were stored at -80ºC until sectioning. Tissues were sliced into 8 micron sections onto Vectabond (Vector Laboratories, Burlingame, CA) treated slides, dried for 10 m, and fixed in 95% ethanol at 4ºC for 10 m. The slides were washed for 5 m in PBS and then blocked for 1 h in 10% normal donkey serum at ambient temperature. The slides were incubated in 2a11, rat anti-F4-80 (Caltag), rat anti-
neutrophil (Serotec) or isotype control antibodies (10 µg/ml in 1% BSA in PBS) for 1 h at 4°C followed by washing (3x) for 5 m in PBS. The slides were incubated in AF488 (Molecular Probes) or Texas Red (Jackson ImmunoResearch, West Grove, PA) conjugated anti-rat antibody (10 µg/ml) and 300 nM DAPI (Molecular Probes) for 1 h at ambient temperature. The slides were washed 2 times for 5 m in PBS, and then once for 5 m in water. The slides were mounted under cover slips with Prolong Anti-Fade (Molecular Probes) and imaged with a fluorescent microscope.

Confocal Microscopy

All slides were evaluated on a Leica DM IRBE inverted confocal microscope with the TC2 SP2 microscope system (Leica, Exton, PA).

**In Vivo Internalization Studies.** Resident peritoneal cells, blood, and spleens were harvested from mice at 3 and 5 h after injection with AF488 labeled GP. The cells were prepared as described for flow cytometry, except that after lysis of red blood cells the cells were fixed in 4% w/v paraformaldehyde (Fisher Scientific) at ambient temperature for 10 m. The cellular nuclei were counterstained by incubating the cells with 1 µg/ml propidium iodide (Molecular Probes) for 10 m. The cells were mounted onto glass slides and cover-slipped using Prolong Anti-Fade mounting media (Molecular Probes). All procedures were performed in the dark.
**In Vitro Co-localization Studies.** Thioglycollate elicited macrophages were cultured in 4 chamber slides (Lab-Tek, Nalge Nunc International. Naperville, IL) at 100,000 cells per well in RPMI-1640 medium supplemented with 10% serum and antibiotics. The remaining procedures were performed in the dark. Cells were incubated with 100 µg/ml fluorescent labeled GP, laminarin, or particulate GP (AF647) in serum free media at 4°C for 3 hrs, then at 37°C for 5-60 m. The slides were washed with PBS, fixed with 4% paraformaldehyde (Fisher Scientific), and permeablized with 1% Triton-X100 (Sigma). The cells were blocked (1 h) with 10% normal horse serum and then incubated overnight with goat anti-Dectin-1 antibody (R and D Systems), or with 1 of the following antibodies from Santa Cruz Biotechnology (Santa Cruz, CA): goat anti-GM130, goat anti-lamp1, goat anti-GRP78, goat anti-TLR2, goat anti-scavenger receptor A, rabbit anti-caveolin-1, rabbit anti-clathrin heavy chain, rabbit anti-rab5B, rabbit anti-P85 at 4°C. Alternately, cells were stained for lipid rafts using an AF 555 Vybrant Lipid Raft Labeling Kit (Molecular Probes) according to manufacturer’s directions. Following 3 washes with PBS, the cells were stained with rhodamine (TRITC) conjugated anti-goat antibody (Jackson ImmunoResearch) or AF 546 conjugated anti-rabbit antibody (Molecular Probes) diluted in Sytox green nuclear counterstain (Molecular Probes). The slides were washed in PBS, followed by water, and then cover-slips were mounted using Prolong Anti-fade (Molecular Probes).

Images were evaluated using multicolor/2D cytofluorogram software from Leica Microsystems. The software quantifies the extent of co-localization by creation of a binary mask of the image data in the cytofluorogram (Figure 10). The binary mask is created by masking all of the pixels that are double positive for both the GP fluorescence and for the organelle or receptor fluorescence. Co-localization was then assessed using the mask intensity rate for the co-localized GP versus the overall intensities of the GP in the image. A rate distribution analysis
was performed for each type of glucan. Two peaks were noted with each type of glucan and were denoted negative and positive for co-localization. Thus mask intensity rates of $\geq 35\%$ were considered positive for co-localization for soluble glucans (Figure 11A) and $\geq 20\%$ was considered co-localized for particulates (Figure 11B).

Figure 10  Representative 2-D cytofluorogram  The green pixels represent one fluor, i.e. the anti-organelle antibody, in the image while the red pixels represent the other fluor (i.e. fluorescent labeled glucan). The blue pixels represent the co-localized fluorors. The mask intensity rate is calculated as the glucan pixels in the co-localized area as a percentage of all glucan pixels in the image.
Figure 11 Frequency of mask intensity rates for soluble (A) and particulate (B) glucans. Mask intensity rates were determined for individual glucan containing cells as described. The values were rounded to the nearest 5 (i.e. 5, 10, 15), and the frequency of each value was determined. Based on this analysis, a mask intensity rate greater than 35 was determined to be positive for co-localization for soluble glucans and greater than 20% was considered positive for particulate glucan.

Statistical Analysis

Percent Dectin-1 positive cells, percent neutrophils, and glucan fluorescence data were summarized by the mean and standard error (SEM). Group mean responses were compared by analysis of variance and pair-wise multiple comparison testing by the least significant difference procedure (or Tukey’s procedure when ANOVA was not significant). Probability levels of 0.05 or smaller were considered significant.
CHAPTER 4

RESULTS

Mortality Trend of Mice Infected with *C. albicans*

To address Aim 1 a model of systemic fungal infection in mice was required. *C. albicans* was chosen as it is the most common opportunistic fungal pathogen (Wilson et al. 2002; Sullivan et al. 2004). The strain of *C. albicans* used in this study is a human vaginal isolate that has been used as a laboratory control strain since the early 1980s. No information on the effects of this strain was available in mice. To establish the appropriate challenge dose, groups of mice were injected IV with *C. albicans* blastospores over a dose range of $1 \times 10^5$ to $1 \times 10^7$ cfu/mouse. Doses above $1 \times 10^5$ cfu/mouse resulted in rapid mortality, usually within 2-3 days (data not shown). Extensive survival analysis using $1 \times 10^5$ cfu/mouse of *C. albicans* was then performed. Intravenous injection of $1 \times 10^5$ cfu/mouse of *C. albicans* resulted in a median survival time (MST) of 13 days and time to 100% mortality of 21 days (Figure 12). Based on the survival kinetics, samples were collected on days 1, 3, 5, 7, and 14 of infection (Figure 12, arrows).
Figure 12 Mortality trend for *C. albicans* infection  Mice were injected at time 0 with $1 \times 10^5$ cfu *C. albicans*, IV. The first death was recorded on day 6. The median survival time was 13 days and time to 100% mortality was 21 days. Arrows indicate the time intervals at which samples were harvested. N=10.

**Increased Dectin-1 Expression in *C. albicans* Infection is Primarily Because of Increased Recruitment of Dectin-1 Positive Cells into the Blood and Spleen**

Previous studies regarding the interaction of *C. albicans* and Dectin-1 have been performed *in vitro* (Brown and Gordon 2001; Brown et al. 2003; Gantner et al. 2005). Thus the effects of systemic infection with *C. albicans* on Dectin-1 *in vivo* have not been determined. Dectin-1 expression on blood and splenic leukocytes from *C. albicans* infected and control mice were compared by flow cytometry. Overall, the percentage of Dectin-1 positive cells was increased in both blood and splenic leukocytes from *C. albicans* infected mice when compared to
Figure 13  Infection with *C. albicans* increases Dectin-1 positive cells in the blood and spleen. Mice were injected at time 0 with $1 \times 10^5$ cfu *C. albicans*, IV. Blood (A and C) and spleen (B and D) cells were harvested and stained with anti-Dectin-1 antibody (2a11) prior to flow cytometric analysis. A and B. Representative histograms of 3 and 7 d data, respectively. C and D. The data were normalized to the unchallenged control group, which was set at 1. N=3/group/time interval. values are means ± SEM, * indicates p<0.05 compared to control at each time point.

non-infected controls (Figures 13A and B). Increases of 44.0%, 23.5%, and 58.9% (p<0.05) were observed in the percentage of Dectin-1 positive blood leukocytes on days 3, 5, and 14, respectively (Figure 13C). The percentage of Dectin-1 expressing splenic leukocytes showed an 86.5% (p<0.05) increase at day 7 post-infection (Figure 13D). The increase seemed to be biphasic with peaks at 3 and 7 days. Dectin-1 expression has been reported to vary among the
different leukocyte cell types (Willment et al. 2001; Brown et al. 2002; Taylor et al. 2002). Therefore, 4 color flow analysis was performed to determine cell type and Dectin-1 expression in order to determine whether the increases in Dectin-1 expression were because of changes in cellular population dynamics or because of changes in Dectin-1 surface expression on individual cells. *C. albicans* infection increased the percentage of neutrophils and decreased the percentage of T lymphocytes in both the blood and the spleen (Figure 14). In the blood the percentage of neutrophils was significantly increased by 107.3%, 98.3%, and 76.2% at 3, 5, and 14 days, respectively (Figure 14A). The percentage of splenic neutrophils was increased by 353% and 215.9% (p<0.05) at 7 and 14 days, respectively (Figure 14B). In the blood the percentage of monocytes was increased on day 3 post-infection (data not shown). Conversely, the percentage of T lymphocytes was decreased in both the blood (Figure 14C) and in the spleen (Figure 14D), when compared to non-infected control mice. A decrease of 37.9%, 34.0%, and 65.7% (p<0.05) in the percentage of blood T cells was observed on days 3, 5, and 7 post-infection, respectively (Figure 14C). The percentage of splenic T cells was decreased by 35.0% (p<0.05) on day 14 post-infection (Figure 14D).

The Overall Amount of Dectin-1 Expressed by Neutrophils is Decreased, but the Percentage of Dectin-1 Positive T Cells is Increased, in Response to Systemic Candidiasis

Neutrophils are felt to be the first line of defense against *C. albicans* (Romani 2004) and express high levels of Dectin-1 on their surface (Taylor et al. 2002). However, infection with *C. albicans* resulted in a decrease in the amount of Dectin-1 expressed on the surface of both blood and splenic neutrophils (Figures 15A and B), though the percentage of Dectin-1 positive
Figure 14 C. albicans infection increases the percentage of blood and splenic neutrophils and a decrease in blood and splenic T lymphocytes. Mice were injected at time 0 with $1 \times 10^5$ cfu C. albicans, IV. Blood leukocytes (A and C) and splenocytes (B and D) were harvested and stained with anti-neutrophil (A and B) and anti-T lymphocyte (C and D) antibodies, prior to flow cytometric analysis. The data were normalized to the unchallenged control group, which was set at 1. N=3/group/time interval. Values are means ± SEM, *indicates p<0.05 compared to control at each time point.

neutrophils remained unchanged (data not shown). On days 7 and 14 post-infection, Dectin-1 mean fluorescence on blood neutrophils was decreased by 52.61 and 50.23%, respectively (p<0.05) (Figure 15A). Splenic neutrophils also showed decreased Dectin-1 mean fluorescence that was significant on days 3 and 7 post-infection (48.96 and 53.28%, respectively, p<0.05).
(Figure 15B). Typically, T cells have been found to express Dectin-1 at low levels, if at all (Taylor et al. 2002). While overall the percentage of T cells in the spleen decreased in response to candidiasis, the remaining T cells showed an increased percentage of Dectin-1 positive cells on days 3 (36.6%) and 7 (23.3%) when compared to control mice (p<0.05) (Figure 15C). Thus, the increase in Dectin-1 positive cells in the blood and spleen of mice with candidiasis is primarily because of an increased recruitment of Dectin-1 positive neutrophils to the periphery, although there was a decrease in neutrophil Dectin-1 surface expression. In contrast, we observed a decrease in the percentage of T cells, but the percentage of those T cells that were Dectin-1 positive was increased in response to systemic *C. albicans* infection.

Figure 15  Neutrophils expressed less Dectin-1 on their surface with *C. albicans* infection, while an increased percentage of Dectin-1 positive T cells was observed in the spleens of mice with *C. albicans* infection. Mice were injected at time 0 with 1 x 10^5 cfu *C. albicans*, IV. Blood leukocytes (A) and splenocytes (B and C) were harvested and stained with anti-neutrophil (A and B), anti-T lymphocyte (C), and anti-Dectin-1 (2a11) prior to flow cytometric analysis. The mean fluorescence for Dectin-1 was determined on the neutrophil population and the percent Dectin-1 positive cells was determined in the T lymphocyte population. The data were normalized to the unchallenged control group, which was set at 1. N=3/group/time interval. Values are means ± SEM, * indicates p<0.05 compared to control at each time point.
Dectin-1 Positive Neutrophils and Macrophages are Recruited into \textit{C. albicans} Induced Renal Lesions

Previous studies have reported that systemic challenge of mice with \textit{C. albicans} results in the development of renal abscesses (Rogers and Balish 1978; Williams et al. 1978). To confirm that the present model of candidiasis was nephrotrophic, disease progression was characterized by histopathology and culture of \textit{C. albicans} in tissues. Blood, kidney, and lung tissues were cultured at 1, 3, 5 and 7 days post-infection. \textit{C. albicans} was cultured from all infected mice at all time intervals (Table 1). Blood cultures were positive only on day 1, although large numbers of \textit{C. albicans} were cultured from all kidneys at all time intervals (Table 1). Lung tissue showed sporadic culture positivity. Control tissues were negative for growth at all time intervals (data not shown). The lack of positive blood cultures, despite highly positive cultures from other organs, is consistent with findings from patients in which up to 40\% of those with wide-spread infection are blood culture negative (Bodey 2000).

Table 1 Presence of viable \textit{C. albicans} in blood, kidney and lung

<table>
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<td>Lung</td>
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Tissue cultures were carried out as described. Each quadrant positive for candidal growth is represented by +, whereas, - indicates no growth. N=3/group/time point.
Systemic infection with *C. albicans* induces renal abscesses in mice. Mice were injected on day 0 with $1 \times 10^5$ cfu *C. albicans* or diluent. Kidneys were harvested on day 3 post-challenge. H and E (A) staining showed renal lesions surrounded by inflammatory infiltrate (dark blue, 100X). Periodic acid Schiff staining (B) of a consecutive section clearly demonstrates the presence of fungal elements characterized by a hyphal mass (red, 100X).

Renal abscesses were grossly apparent upon necropsy (data not shown). To better characterize the colonization of the kidney, sections were fixed and stained by H&E and PAS (Figure 16). H&E sections of kidney show multiple zones of proliferation of *C. albicans* (lavender) with associated inflammatory infiltrate (deep blue) (Figure 16A). PAS staining better illustrates the proliferation of fungal organisms (red) (Figure 16B). Fungal hyphae were clearly evident and foci of candidal proliferation were present in the renal cortex and medulla. We conclude that this strain of *C. albicans* is nephrotrophic.

Because the kidneys were the primary organ affected by *C. albicans* infection, the level of Dectin-1 expression in renal foci was determined by performing immunohistochemistry on the kidneys from infected and non-infected mice. As expected, overall Dectin-1 expression (green) in the *C. albicans* infected kidneys was increased in the area of renal lesions (Figure 17B, white arrows) when compared to kidneys from normal control mice (Figure 17A). Dectin-1 expressing
cells were found near glomeruli and in the nearby tubular system. Higher magnification shows the Dectin-1 positive cells (Figure 17D, white arrows) associated with foci of fungal elements (Figure 17D, arrowhead), while the control kidney shows very little Dectin-1 positivity (Figure 17C). Staining of sequential slices for macrophages and neutrophils revealed that the area of Dectin-1 positivity was co-localized to an area containing predominantly macrophages (in red) (Figure 18A) with fewer neutrophils (in red) (Figure 18B). Fungal elements are again evident in these sections (Figure 18, arrowheads). Thus, the increased Dectin-1 expression observed in the kidneys of mice with candidiasis is because of an influx of Dectin-1 positive macrophages and, to a lesser extent, neutrophils in areas immediately adjacent to the candidal elements.
Dectin-1 positive cells are found in association with *C. albicans* induced renal lesions. Mice were injected on day 0 with $1 \times 10^5$ cfu *C. albicans* or diluent. Immunohistochemistry using anti-Dectin-1 antibody (green) and the DAPI nuclear counterstain (blue) showed Dectin-1 positivity (white arrows) in kidneys from *Candida* infected mice (B). Control kidneys (A) did not show Dectin-1 positivity (100X). Higher magnification (400X) revealed that while the control kidney was not positive (C), in the *Candida* infected kidney, Dectin-1 positive areas (D, white arrows) were associated with fungal elements (D, arrowhead). Representative images.
Figure 18  Dectin-1 positive macrophages and neutrophils are found in association with *C. albicans* induced renal lesions. Mice were injected on day 0 with $1 \times 10^5$ cfu *C. albicans* or diluent. Staining of consecutive sections from those in Figure using anti-macrophage or neutrophils antibody (red) and the DAPI nuclear counterstain (blue) showed that the Dectin-1 positive areas predominantly co-localized with macrophages (red) (A) and to a lesser extent with neutrophils (red) (B). These cells are also adjacent to fungal elements (arrowheads). 400X. Representative images.

Mortality Trend of Mice With Cecal Ligation and Puncture Induced Polymicrobial Sepsis in the Presence and Absence of Glucan Treatment

The previous sections determined the effect of fungal infection on Dectin-1 and completed Aim 1. To address Aim 2 the cecal ligation and puncture model of polymicrobial sepsis was used. Previous studies have shown that this model of polymicrobial sepsis generally has a survival rate of 20%, while immunoprophylaxis with glucan phosphate will improve survival to 80% (Williams et al. 1999b). To confirm that observation and to determine appropriate time intervals for study, ICR/HSD mice were treated with glucan phosphate (40 mg/kg, IP) 1 hr prior to CLP. A single prophylactic injection resulted in a 70% long-term survival rate (p<0.05) (Figure 19). In contrast, control mice subjected to CLP showed a median survival time of ~30 h and a 20% long-term survival (Figure 19). The first deaths occurred in
the control group at ~22 h and in the glucan treated at ~28 h after CLP, respectively. Samples for Dectin-1 analysis were harvested at 3, 6, 12, and 24 h after surgery (Figure 19).

![Graph showing survival rates](image)

Figure 19  Glucan administration improves survival in CLP induced sepsis. Mice underwent CLP at time 0. Glucan phosphate (1 mg/mouse) or diluent was administered IP 1 h prior to surgery. Mice were followed for survival to day 14 post-operatively. N=10/group.

The Percentage of Dectin-1 Expressing Blood Leukocytes is Decreased by Polymicrobial Sepsis and Further Decreased by GP Administration

To determine the in vivo effects of polymicrobial sepsis, Dectin-1 expression was assessed on blood leukocytes using flow cytometry at 3 and 12h post-CLP. During the course of these experiments, 3 populations of peripheral blood Dectin-1 expressing leukocytes were identified in normal mice: negative for Dectin-1, low level expressers of Dectin-1, and high level expressers of Dectin-1 (Figure 20A). Approximately 60% of the total peripheral leukocytes expressed no Dectin-1 (Figure 20A). The majority of peripheral blood leukocytes
from control mice that do express Dectin-1 show high levels of Dectin-1 expression (Dectin\(^{\text{high}}\)) (Figure 20). Following CLP and/or glucan administration there is a dramatic and significant shift in Dectin-1 expression levels from the high expression population to the low expression population (Figure 20A). However, Dectin-1 expression is not completely lost from all Dectin-1 expressing cells in the presence of sepsis, glucan, or a combination of the two. Therefore, these leukocytes have been classified as Dectin-1\(^{\text{low}}\), indicating a decrease in Dectin-1 surface expression. For the purposes of this investigation, the data described below focus on the cell populations that show a shift from normal levels of expression (Dectin-1\(^{\text{high}}\)) to decreased Dectin-1 expression (Dectin-1\(^{\text{low}}\)).

Flow cytometric analysis revealed that CLP significantly decreases the percentage of Dectin-1\(^{\text{high}}\) expressing cells by 51.4 and 50.9% at 3h when compared to control and sham surgery, respectively (Figure 20B, p<0.05). Dectin-1\(^{\text{high}}\) expressers returned to baseline by 12 h (Figure 20C). The percentage of Dectin-1\(^{\text{low}}\) expressing cells was increased by CLP at 3 h post-operatively when compared to control (145.9%) and to sham (54.5%) (data not shown). The percentage of Dectin-1\(^{\text{low}}\) positive cells returned to control values by 12 h (data not shown). Glucan administration resulted in a significant decrease of 48.5% in the percentage of Dectin-1\(^{\text{high}}\) positive leukocytes at 3 h post-CLP when compared to untreated CLP animals (Figure 20B, p<0.05). Dectin-1\(^{\text{high}}\) expressers in glucan treated mice returned to control values by 12h post surgery (Figure 20C). The percentage of Dectin-1\(^{\text{low}}\) positive blood leukocytes was increased by 28.5% at 3 h post-CLP in glucan treated animals when compared to CLP alone (data not shown). These cell numbers also returned to normal at 12 h (data not shown).
The Percentage of Dectin-1 Positive Splenic Leukocytes was Decreased while the Number of Dectin-1 Positive Peritoneal Cells was Increased by Polymicrobial Sepsis

Dectin-1 expression on splenic and peritoneal leukocytes was examined using flow cytometry at 0, 3, 6, 12, and 12 h post-CLP. Splenic leukocytes from CLP animals showed a significant decrease in the percentage of Dectin-1 expressing cells (19.6%) when compared to Sham, but this decrease was only significant at 12h (Figure 21A, p<0.05). In glucan treated septic mice, the percentage of Dectin-1 expressing cells was significantly decreased in the spleen at 6 and 12h post-CLP by 33.9 and 34.1%, respectively when compared to untreated CLP mice (Figure 21B, p<0.05).

Figure 20  Sepsis decreases Dectin-1 expression on peripheral leukocytes The administration of glucan results in a further decrement in leukocyte Dectin-1 positivity. Mice underwent no surgery (control), lapartomy (sham) control or CLP at time 0. Glucan phosphate was administered 1 h prior to surgery. Peripheral leukocytes were isolated and assayed for Dectin-1 surface expression at 3 (A and B) and 12 h (C). A. Representative histogram of 3 h data. B and C. Data are normalized to the no surgery control, which was set at 1.0. N=3/group, values are means ± SEM. *p<0.05 compared to control; + p<0.05 compared to sham surgery and # p<0.05 compared to CLP.
Figure 21  Sepsis decreases the percentage of Dectin-1 positive splenocytes, and glucan further decreases this percentage. Mice underwent sham or CLP at time 0. Diluent or glucan was injected 1 h prior to surgery. At 0, 3, 6, 12, and 24 h spleens were harvested, reduced to single cells, and stained with anti-Dectin-1 antibodies. Data were normalized to 0 h means which were set to 1. N=3/group/time point, values are means ± SEM. *indicates p<0.05 compared to sham (A) or to CLP (B).

The percentage of Dectin-1 positive peritoneal leukocytes from mice that have undergone CLP showed a significant 774% increase 12h post-operatively when compared to sham surgery mice (Figure 22A, p<0.05). Glucan treatment with CLP significant decreased the percentage of Dectin-1 expressing cells at 3 (89.2%) and 12h (90.1%) when compared to CLP alone (Figure 22B).
Figure 22  Sepsis increases Dectin-1 expressing resident peritoneal cells, while glucan administration with sepsis causes a decrease in Dectin-1 positive resident peritoneal cells. Mice underwent sham surgery or CLP at time 0. Glucan phosphate was administered 1 h prior to surgery. At 0, 3, 6, 12, and 24h resident peritoneal cells were harvested and assayed for Dectin-1 expression by flow cytometry. Data were normalized to 0 h means. N=3/group/time point values are means ± SEM. * indicates p<0.05 compared to sham (A) and CLP (B).

Changes in Overall Dectin-1 Expression in CLP Are Because of Changes in Cellular Dectin-1 Expression and Changes in Cell Populations

As with fungal sepsis 4 color analysis was performed to determine if changes in Dectin-1 expressing cells were solely because of changes in Dectin-1 expression on individual cell types and/or the result of changes in cell population dynamics in response to sepsis and/or glucan treatment. We found that sepsis and/or glucan treatment resulted in a decrease in the percentage of Dectin-1 positive blood neutrophils (Figure 23A and B). Specifically, the percentage of Dectin-1 positive neutrophils was decreased at 3 h and 12 h after CLP when compared to control and sham surgery (Figure 23B). The effect persisted at 12 h post-CLP (53.2 and 39.0%, p<0.05) (Figure 23C). Glucan treated CLP mice showed a 51.8% decrease (p<0.05) in the percentage of Dectin-1 positive blood neutrophils when compared to CLP alone at 3 hr post-operatively.
(Figure 23B). However, the effect was lost by 12 h (Figure 23C). While the percentage of Dectin-1 positive neutrophils decreased, there was no significant change in the total percentages of blood neutrophil, monocyte, or lymphocyte cells, or in the percentage of Dectin-1 positive monocytes or lymphocytes in response to CLP or to glucan treatment with CLP (data not shown). Thus, we conclude that the decrease in Dectin-1 positive blood leukocytes in CLP and in glucan treated CLP animals is because of a decrease in Dectin-1 surface expression in neutrophils, not a change in the total percentage of neutrophils.

In the spleen there was an 11.2% (p<0.05) decrease in the percentage of macrophages in CLP mice when compared to sham surgery controls 6 hr post-CLP (Figure 24A). However, there was no change in the percentage of splenic Dectin-1 positive neutrophils, macrophages, or lymphocytes (data not shown). Therefore, the decrease in splenic cells expressing Dectin-1 in
CLP animals is because of a shift in cell populations rather than because of changes in Dectin-1 expression by the various cell types. With glucan treatment, CLP mice showed a significant increase of 76.0% in the percentage of Dectin-1 positive splenic macrophages at 3 h and a decrease of 43.5 and 51.6% in the percentage of macrophage expressing Dectin-1 at 6 and 12 h, respectively, when compared to untreated CLP mice (p<0.05) (Figure 24B). The percentage of Dectin-1 expressing splenic neutrophils were significantly increased at 12 h (47.2%, p<0.05) (Figure 24C). There was no significant shift in cell populations at either time interval nor was there any change in the percentage of Dectin-1 expressing splenic lymphocytes (data not shown).

We conclude that the decrease in overall splenic Dectin-1 positive cells with glucan treatment and sepsis (12 hr) is because of a decrease in the percentage of Dectin-1 positive splenic macrophages.

Figure 24  The decrease in the percentage of Dectin-1 positive splenocytes observed during sepsis is because of a reduction in the overall percentage of macrophages. With glucan treatment, the further decrease in the percentage of Dectin-1 positive splenocytes is because of a decrease in the percentage of Dectin-1 positive macrophages. Mice underwent sham or CLP at time 0. Glucan phosphate was administered 1 h prior to surgery. At 0, 3, 6, 12, and 24h spleens were harvested, reduced to single cells, stained with anti- macrophage (A and B.), neutrophil (C.), and/or Dectin-1 (B and C.) antibodies, and analyzed by flow cytometry. Data were normalized to 0h means which were set to 1. N=3/group/time point, values are means ± SEM. * indicates p<0.05 compared to CLP.
C. albicans and S. cerevisiae Glucans Decrease Dectin-1 Expression on Primary Neutrophils In Vitro

In an effort to elucidate the mediators inducing the changes in Dectin-1 surface expression on circulating neutrophils in vivo with fungal and polymicrobial sepsis, thioglycollate elicited neutrophils were cultured with S. cerevisiae GP, C. albicans blastospore GP, C. albicans hyphal GP, LPS, mannan, or combinations of the different PAMPs. The cells were evaluated for Dectin-1 expression by flow cytometry. The GP from all sources resulted in a loss of Dectin-1 from the neutrophil cell surface. The S. cerevisiae GP reduced the percentage of Dectin-1 positive cells by 51.02% (p<0.05) (Figure 25). The C. albicans GP had similar effects on Dectin-1 expression with the blastospore GP decreasing the percentage of Dectin-1 positive cells by 57.63% (p<0.05) and the hyphal GP decreasing it by 54.22% (p<0.05) (Figure 25). Neither the LPS alone nor the mannan alone significantly altered the expression on the cell surface, but the combination of LPS with S. cerevisiae GP and the combination of mannan with the C. albicans blastospore GP resulted in a reduction in the percentage of neutrophils expressing Dectin-1 (Figure 25). The LPS/GP combination reduced the percentage of Dectin-1 expressing cells by 32.77% (p<0.05) and the mannan/GP combination reduced it by 57.15% (p<0.05) (Figure 25). It can be concluded from these data that the loss of Dectin-1 from the neutrophil surface with candidiasis is because of GP released from either the blastospore or hyphal form. In polymicrobial sepsis, the loss of Dectin-1 positive cells is not because of the effect of LPS, but may be because of circulating glucans released during sepsis (Digby et al. 2003). However, the further loss of these cells with GP treatment is because of the effect of GP on Dectin-1.
Figure 25  *S. cerevisiae* and *C. albicans* derived glucans reduce the percentage of Dectin-1 expressing elicited neutrophils alone and in combination with other PAMPs. Thioglycollate elicited neutrophils were incubated with 1 µg/ml *S. cerevisiae* GP, *C. albicans* blastospore or hyphal GP, LPS, or *S. cerevisiae* mannan alone or in combination for 3 h. The cells were stained with anti-Dectin-1 and anti-neutrophil antibodies and analyzed by flow cytometry. The percentage of Dectin-1 positive cells within the neutrophil positive population was determined. Average of 3 independent experiments with N=6, values are means ± SEM. * indicates p<0.05 compared to control.

Systemically Administered GP is Internalized by Blood, Splenic, and Peritoneal Leukocytes

The previous studies have determined that systemic infections modulate Dectin-1 expression. Additionally, the polymicrobial sepsis studies suggested that GP administration alone will also modulate Dectin-1; therefore Aim 3, focuses on the effect of systemic glucan administration on Dectin-1. Though prior studies have shown that GP is internalized by Dectin-1 expressing cells *in vitro* (Mueller et al. 1996; Herre et al. 2004), and we have shown that GP has biologic activity *in vivo* (Williams et al. 1978; Williams et al. 1991b; Williams et al. 1999b;
Williams et al. 2004a), previous studies have not determined if glucan is internalized by circulating leukocytes \textit{in vivo}. To address this issue, blood, splenic, and peritoneal leukocytes were harvested 5h after administration of fluorescent labeled GP using confocal microscopy. Five hours after IV administration, labeled glucan (green) could be seen within 1.86% of blood leukocytes, 93.67% of which were polymorphonuclear cells with the remainder being mononuclear cells (Figure 26). Fluorescent labeled glucan was also detected within 1.91% splenic leukocytes (Figure 26). Labeled glucan was also visible in the blood and spleen 5h after IP administration of fluorescent glucan (data not shown). Five hours after IP administration of labeled GP, glucan could be detected in 86.20% of peritoneal leukocytes (Figure 26). This indicates that systemically administered glucans are uptaken by circulating and tissue residing leukocytes.
Figure 26  GP is internalized *in vivo* by blood, splenic, and peritoneal leukocytes. Mice were injected with fluorescent labeled GP (green, 5 mg/mouse) either IV or IP. At 5 h after injection, blood, spleen and peritoneal leukocytes were harvested, counterstained with PI (red), and imaged using confocal microscopy.

**Parenteral Administration of GP *In Vivo* Induces a Prolonged Decrease in the Percentage of Dectin-1 Expressing Blood Leukocytes**

The previous experiment determined that glucans are internalized by circulating leukocytes. To determine the long-term effect of glucans on Dectin-1 in these cells, Dectin-1
expression on mouse blood leukocytes was determined by flow cytometry 3 h, 1, 3, 5, 7, and 10 d after IV and IP administration of GP. Administration of GP resulted in a significant loss of Dectin-1 from peripheral blood leukocyte membranes (Figures 27A and B). The Dectin-1\textsuperscript{high} blood leukocytes were decreased by 42.8, 62.9, 83.1, 38.0, and 51.3% at 3h, 1, 3, 5, and 7d, respectively, with IV glucan when compared to control animals (p<0.05) (Figure 27B). The percentage of Dectin-1\textsuperscript{high} blood cells were significantly decreased by 81.0, 54.8, 47.3, and 39.5% with IP glucan administration compared to control animals at 3 h, 1, 5, and 7 d, respectively (p<0.05) (Figure 27B). These results are similar to those found with IV glucan administration. Conversely, GP caused a significant increase in the percentage of Dectin-1\textsuperscript{low} blood leukocytes when compared to controls (data not shown). This likely represents a shift from the Dectin-1\textsuperscript{high} phenotype to the Dectin-1\textsuperscript{low} phenotype because of internalization of Dectin-1. The increase with IV administration was 74.1, 157.8, and 54.9% on days 1, 3, and 5 after injection, respectively, and 52.2, 204.2, and 37.6 on days 1, 3, and 5 after IP injection, respectively (p<0.05) (data not shown). The percentage of both Dectin-1\textsuperscript{high} and Dectin-1\textsuperscript{low} cells returned to control levels by 7 days after injection in both IV and IP treated animals (Figure 27B and data not shown).
Figure 27  GP treatment decreases the number of Dectin\textsuperscript{high} positive blood leukocytes and peritoneal leukocytes. Mice were injected with GP (1 mg/mouse) either IV or IP. At 0, 3, 6, 12 h and 1, 3, 5, 7, and 10 d after injection blood and peritoneal cells were harvested, stained with anti-Dectin-1 antibody, and analyzed by flow cytometry. A. Representative histogram of 3 d data. B and C. Percent positive cells have been represented as a percentage of the control mean at each time point. N=3/group/time, values are means ± SEM. * indicates p<0.05 comparing IV glucan to control and + indicates p<0.05 IP glucan compared to control.

Intraperitoneal administration of glucan causes a substantial decrease in the percentage of Dectin positive peritoneal leukocytes, but this decrease is transient when compared to the changes found in the blood. The percentage of Dectin positive peritoneal leukocytes was decreased by 63.1, 61.9, and 47.8% at 3, 6, and 12 h after IP injection and returned to normal levels by 24 h when compared to control mice (P<0.05) (Figure 27C). Administration of GP also decreased the percentage of Dectin positive splenic leukocytes; however, a decrease of 18.1% is significant only with IP administration on day 1 post-injection when compared to control (p<0.05) (data not shown).
Administration of GP Induces a Prolonged Loss of Dectin-1 Positivity in Peripheral Blood Neutrophils and Monocytes

As with the sepsis studies, the cells responsible for the changes in overall Dectin-1 expression were identified by 4 color flow cytometry. Though there was a trend toward neutrophilia with both IV and IP injection of glucan, this increase was only significant with IV administration at 3 h (data not shown). Neither IP nor IV administration of GP had an effect on the percentage of monocytes in the overall blood leukocyte population at any time interval compared to control (data not shown). However, glucan administration affected the percentage of Dectin-1 expressing neutrophils and monocytes. Specifically, GP decreased the percentage of Dectin-1 expressing blood neutrophils (Figure 28A) and monocytes. IV administration of glucan caused significant decreases of 49.5, 82.5, 64.3, 49.0% in the percentage of Dectin expressing blood neutrophils at 1, 3, 5, and 7 d after injection (Figure 28B), respectively, and significant decreases of 39.0, 74.5, and 33.4% in the percentage of Dectin-1 expressing blood monocytes at 1, 3, and 5 d after injection (Figure 28C), respectively, when compared to control animals (p<0.05). Administration of GP IP caused decreases of 84.9, 57.3, 64.6% in the percentage of Dectin-1 expressing blood neutrophils at 3 h, 3 d, and 5 d post-injection (Figure 28B), respectively, and decreased Dectin-1 expressing blood monocytes by 36.4, 34.6, 48.2% at 3h, 1d, and 3d post-injection (Figure 28C), respectively, when compared to controls (p<0.05).
Figure 28  GP treatment decreases the number of Dectin-1 positive blood neutrophils and monocytes  Mice were injected with GP (1 mg/mouse) either IV or IP. Blood was harvested, stained with anti-Dectin-1, anti-neutrophil, and anti-monocyte antibodies, and analyzed by flow cytometry. Histograms were gated to the neutrophil (A and B) or the monocyte (C) population and the percentage of Dectin-1 positive cells was determined. A. Representative histogram of 3 d neutrophil data. B and C. Percent positive cells have been expressed as a percentage of the control mean at each time point. N=3/group/time, values are means ± SEM. * indicates p<0.05 comparing IV glucan to control and + indicates p<0.05 IP glucan compared to control.

Administration of GP Does Not Alter Membrane Dectin-1 Expression on Bone Marrow Precursors

The data shown above demonstrate that GP will decrease the percentage of neutrophils and monocytes expressing membrane Dectin-1. However, neutrophils and monocytes have a circulating half-life of hours to a few days. In addition, Rice et al. have reported that parenterally administered GP has a plasma elimination half-life of 3.8 h (Rice et al. 2004).
Therefore, an important question was how a single injection of GP could result in such a prolonged loss of leukocyte membrane Dectin-1? One possibility is that GP may also act at the level of leukocyte bone marrow precursors. To answer this question, bone marrow cells were harvested from mice 1, 3, 5, 7, and 10 d after treatment with GP and evaluated for Dectin-1 expression by flow cytometry. GP administration had no significant effect on the percentage of Dectin expressing bone marrow cells when compared to control animals at any time interval (data not shown). Therefore, Dectin-1 levels do not appear to be decreased in bone marrow precursors prior to their entry into the systemic circulation.

The Effect of GP on Loss of Leukocyte Dectin-1 Levels is Specific and Not Observed with Non-glucan Carbohydrate Polymers

To examine the specificity of glucan on leukocyte membrane Dectin-1 expression, mice were injected with mannan, a fungal cell wall mannose polymer (Chauhan et al. 2002), and pullulan, an alpha-linked glucose polymer. At 1 and 5 d after injection blood leukocytes were harvested and Dectin-1 expression and leukocyte phenotype was assessed using flow cytometry. As expected, GP resulted in significantly lower levels of leukocyte Dectin-1 when compared to controls (Figure 29A). However, administration of mannan increased the percentage of Dectin-1 expressing cells by 35.0 and 55.4% on days 1 and 5 after injection when compared to control animals (p<0.05) (Figure 29A).

Leukocyte phenotypic analysis revealed that mannan increased the percentage of Dectin expressing blood neutrophils by 5.8 and 3.1% (Figure 29B) and the percentage of Dectin expressing blood monocytes by 8.9 and 8.4% at 1 and 5d, respectively, when compared to
controls (p<0.05) (Figure 29C). GP again resulted in a significant decrement in the percentage of Dectin-1 expressing neutrophils and monocytes (Figures 29B and C, respectively).

Unlike GP, mannan administration significantly increased the percentage of neutrophils and monocytes. The percentage of blood neutrophils was increased by 70.0% at 5 d after injection when compared to untreated controls (p<0.05) (Figure 30A). The percentage of monocytes was elevated by 51.7% at 5 d when compared to untreated animals (p<0.05) (Figure 30B). Therefore, the increase Dectin positive blood leukocytes at 5 d is because both of an increase in Dectin positive neutrophils and monocytes and an increase in neutrophils and monocytes, cells known to express the highest levels of Dectin-1 on their surface. Pullulan had
very little or no effect on the make-up of the cell population or the percentage of Dectin-1 positive blood leukocytes at either time interval (Figure 30). These results indicated that the effect of GP on Dectin expressing blood leukocytes is specific to carbohydrates known to interact with the Dectin-1 receptor.

The Effect of Glucans on the Loss of Leukocyte Dectin-1 Levels is Dependent on the Type of Glucan

Glucans are found in the cells walls of not just fungi but also other microbes as well as plants. Not all of these glucans are biologically active (Lowe et al. 2001). Therefore, to determine if the effect of GP on Dectin-1 was specific to biologically active glucans, mice were injected with laminarin, a smaller molecular weight β-glucan that does not stimulate intracellular signaling (Lowe et al. 2001) and Dectin-1 expression and phenotype of blood leukocytes was
measured at 3 h and 1 d after injection. Laminarin also resulted in a decrease in the percentage of Dectin-1$^{\text{high}}$ expressing blood leukocytes, but the effect was much more transient than that seen with GP. Specifically, laminarin resulted in a 47.4% decrease in Dectin-1$^{\text{high}}$ expressing and a 72.1% increase in Dectin-1$^{\text{low}}$ expressing blood leukocytes when compared to control animals (p<0.05) (Figure 31A and data not shown). However, by 24 h after injection, the number of Dectin-1$^{\text{high}}$ positive cells with laminarin treatment had returned to control levels. As with GP, the changes seen in overall Dectin-1 expression with laminarin treatment are because of changes in the percentage of Dectin-1 expressing blood neutrophils (Figure 31B) and monocytes (Figure 31C). At 3 h the percentage of Dectin-1 expressing blood neutrophils is significantly decreased by 23.5% with laminarin (p<0.05) (Figure 31B). By 24 h, however, the percentage of Dectin-1 expressing neutrophils with laminarin treatment is not significantly different from control (Figure 31B). The percentage of Dectin-1 positive blood monocytes was decreased by 22.44% compared to control at 3 h after laminarin injection (p<0.05) (Figure 31C). As with the Dectin-1 positive neutrophils, the percentage of Dectin-1 positive monocytes returns to control levels by 24 h (Figure 31C). From these data we conclude that biologically inactive glucans, represented by laminarin, only transiently affect Dectin-1 expression when compared to a biologically active glucan, GP.
Effect of *C. albicans* Sepsis, Polymicrobial Sepsis, and GP Administration on Cytokines

**In Vivo**

Cytokines and chemokines are felt to play a critical role in stimulating an appropriate immune response to *C. albicans* infection (Mencacci et al. 1998), and the cytokine network is felt to be intimately involved in the pathophysiology of polymicrobial sepsis. Additionally, Willment et al. have reported that GM-CSF, IL4 and IL-13 up regulate Dectin-1 expression, while IL-10 down regulates Dectin-1 expression in *in vitro* cultured macrophages (Willment et al. 2003). For these reasons, changes in 10-16 different circulating cytokines and chemokines were measured in serum from mice with fungal sepsis, polymicrobial sepsis, and GP treatment were evaluated by Luminex analysis.

In mice infected with *C. albicans* the serum concentrations of 16 cytokines and chemokines was measured on days 1, 3, 5, 7, and 14 of infection. We found a significant
elevation in the serum levels of the inflammatory cytokines TNFα and IL-6 in *C. albicans* infected mice (Figure 32). Serum TNFα increased by 49% (p<0.05) at day 14 after infection, when compared to controls (Figure 32A). Serum IL-6 levels in infected mice were increased by 643% at 7 days and by 599% (p<0.05) at 14 days (Figure 32B). In contrast, the anti-inflammatory cytokine IL-10 was significantly decreased by 22% (p<0.05) on day 7 post-infection (Figure 32C). The circulating levels of chemokines were variable throughout the study. The neutrophil chemokine IL-17 (Figure 32D) was increased by 170% at day 14 in infected mice when compared to non-infected controls. MIP-1α, a monocyte, macrophage, and T lymphocyte chemokine, was decreased by 40.2% (p<0.05) at 7 days when compared to control values (Figure 32E). Interestingly, MIG, an IFNγ stimulated chemokine for activated lymphocytes, was increased by 119% (p<0.05) on day 1 (Figure 32F). However, we were unable to detect a difference in IFNγ between control and *C. albicans* infected mice at any time throughout the study, though there was a trend toward elevation on day 1 (data not shown). Additionally, we were unable to detect any change in the systemic concentrations of the remaining Th1 cytokines (IL-12) or Th2 cytokines (IL-4) with *C. albicans* infection (data not shown).
Figure 32 *C. albicans* infection increases serum concentration of pro-inflammatory cytokines and chemokines. Mice were injected on day 0 with $1 \times 10^5$ cfu *C. albicans*. Serum was harvested at various time intervals after fungal challenge and cytokine/chemokines were assayed using a multiplexed bead assay kit. Concentrations were normalized to the control concentrations at each time interval which were set to 1. $N = 5$ group/time interval. Values are means ± SEM. * indicates $p < 0.05$ compared to control.
In mice that underwent CLP, serum was harvested at 12 h post-operatively and analyzed for 10 different cytokines. CLP had a significant effect on the concentration of circulating pro-inflammatory cytokines. Specifically, CLP increased TNFα expression (p<0.01) when compared to the control (533%) and sham (546%) groups, respectively (Figure 33A). IL-6 levels were increased by 190 fold in CLP versus control and 61 fold versus sham (Figure 33B, p<0.01). Likewise, IL-1β levels were increased by 75% in CLP mice relative to the controls (Figure 33C, p<0.02). CLP also increased the anti-inflammatory cytokine IL-10 by 55 fold when compared to the control group (p<0.01) (Figure 33E). There was no difference in the levels of IFNγ, GM-CSF, IL-2, IL-4, or IL-5 between the CLP group and the control and sham groups (data not shown). Administration of glucan to CLP mice did not significantly alter cytokine levels; however, TNFα, INFγ, GM-CSF, IL-2, IL-6, IL-10, and IL-12 levels showed a downward trend when compared to the CLP group (Figure 33 and data not shown). Glucan administration did not alter serum cytokine levels in sham surgery mice, but did reduce IL-2 levels by 11% (p<0.01) in the glucan alone group (Figure 33F).
Figure 33  Polymicrobial sepsis induces production of pro-inflammatory and anti-inflammatory cytokines Mice underwent sham or CLP at time 0. Glucan phosphate (1 mg) or diluent was administered 1 h prior to surgery. Serum was obtained 12 hours postoperatively and assayed using a Luminex multiplex mouse cytokine kit. N=5/group, values are means ± SEM. *indicates p<0.05 compared to control and + indicates p<0.05 compared to sham.
In GP treated mice serum concentrations of 16 cytokines and chemokines were measured at 0, 3, 24, and 72h after IV injection. We found that administration of GP caused a significant increase in the neutrophil chemokines KC and IL-17 24h after injection when compared to 0 h controls. Specifically, KC concentrations were increased by 57.84 % (p<0.05) (Figure 34) and IL-17 concentrations were increased by 2047% (p<0.05) (Figure 34). Interestingly, even though concentrations of INF\(\gamma\) were unaffected by GP administration (data not shown), the concentrations of the interferon induced chemokines IP-10 and MIG were both elevated 3h after glucan treatment. IP-10 was increased by 290.9% (p<0.05) (Figure 34) and MIG was increased by 687.2% (p<0.05) (Figure 34) when compared to 0 h control levels. Concentrations of MCP-1 were also increased by 601.4% 3h after injection when compared to 0h controls (p<0.05) (Figure 34). 24h after injection with GP, IL-12 was decreased by 56.29% (p<0.05) (Figure 34).
Figure 34  GP stimulates granulocyte and monocyte chemokine production while decreasing the immunomodulatory cytokine interleukin 12  Mice were injected with GP (1 mg) IV. At 0, 3, 24, and 72 h after injection serum was harvested and assayed for 16 cytokines and chemokines by Luminex assay. Concentrations were normalized to the 0 h means which were set to 1. N=4 animals/ time point, values are means ± SEM. * indicates p<0.05 compared to 0 h control.
GP Co-Localizes With Dectin-1 upon Internalization

The previous *in vivo* study demonstrates that circulating leukocytes internalize GP, and treatment with GP results in a loss of Dectin-1 from the cell surface. We and others have reported that macrophages internalize Dectin-1 in response to GP treatment (Mueller et al. 1996; Herre et al. 2004). However, there is no evidence to indicate whether the GP and Dectin-1 are internalized and co-localized within the leukocyte. We examined the internalization and co-localization of GP and Dectin-1. Incubation of murine peritoneal macrophages with fluorescent labeled GP resulted in uptake of the GP (green, Figure 35A). In the cells that do not contain glucan, Dectin-1 is primarily membrane associated (red, Figure 35B). In the cell containing GP, the GP co-localized with Dectin-1 in an intracellular vesicle (yellow, Figure 35D). Co-localization with Dectin-1 was found in a mean of 62.16% of cells containing GP (data not shown). We interpret these data to mean that glucans are internalized and co-localized with Dectin-1 in leukocytes.

Figure 35  Co-localization of GP with Dectin-1 in primary murine macrophages
Thioglycollate elicited macrophages were incubated for 10 m with fluorescent labeled GP (A, green), stained with anti-Dectin-1 antibody (B, red), and counterstained with Sytox green nuclear counterstain (C, blue). Images were analyzed by confocal microscopy. Co-localization is shown as yellow (D). Analysis of the co-localized image indicated a mask intensity of >71% which confirms that glucan phosphate co-localizes with Dectin-1 in murine leukocytes.
GP Co-Localizes with Clathrin and Caveolin-1 upon Internalization by Macrophages

Aim 3 determined that Dectin-1 is lost from the cell surface because of internalization of the Dectin-1/glucan complex. In Aim 4 the mechanisms by which the Dectin-1/glucan complex is internalized and trafficked was determined. Aim 4 also addresses any differences in trafficking amongst different types of glucans.

The 2 primary mechanisms for internalization are clathrin mediated and non-clathrin/lipid raft mediated (Le Roy and Wrana, 2005). To determine which of these mechanisms is responsible for Dectin-1/glucan internalization, primary macrophages were incubated with fluorescent labeled glucan (green, Figures 36A and E) and stained with antibodies against clathrin (red, Figure 36B) or caveolin-1 (red, Figure 36F). Caveolin-1 is a tyrosine kinase found in the lipid raft and involved in lipid raft mediated internalization (Le Roy and Wrana, 2005). The cells were analyzed by confocal microscopy. Interestingly, GP co-localized with both clathrin (yellow, Figure 36C) and caveolin-1 (yellow, Figure 36G) after 15 m incubation. Co-localization can better be seen in the masked image (white, Figures 36D and H). The mean percentage of cells positive for co-localization of GP with clathrin was 90.00% and with caveolin-1 was 83.33% at 15 m. Both proteins were also found to co-localize with GP at 5 m (data not shown). At 5 m a mean of 63.89% of cells were positive for co-localization of GP with clathrin, and a mean of 100% were positive for co-localization of GP with caveolin-1. In some cases a receptor can be internalized by either clathrin mediated or caveolin/lipid raft mediated mechanisms (Di Guglielmo et al. 2003); further studies were necessary to determine if this was the case with the Dectin-1/GP complex.
Figure 36  GP co-localized with both caveolin-1 positive vesicles and clathrin positive vesicles. Thioglycollate elicited murine macrophages were incubated with fluorescent labeled GP (green, A and E) for 15 min and then stained with labeled anti-caveolin-1 (red, B) or anti-clathrin heavy chain (red, F) antibody. Co-localization is shown in yellow in the overlay image (C and G) and in white in the masked image (D and H). A mask intensity rate ≥ 35% was considered positive. Representative images.

GP is Internalized by Clathrin Mediated Endocytosis in Macrophages

The previous co-localization study demonstrated that GP is co-localized with both caveolin-1 and clathrin. This suggests that GP is internalized by both clathrin and caveolin/lipid raft mediated mechanisms. To determine if this is the case, macrophages were incubated with hyperosmotic sucrose, which inhibits clathrin mediated uptake, or with MβCD, which depletes cholesterol and inhibits uptake by lipid rafts. Upon treating the cells with fluorescent labeled glucan, we found that the percentage of Dectin-1 positive macrophages was decreased by 18.49% (p<0.05) (Figure 37A) while the mean fluorescence of glucan in the cells was increased by 1431% (p<0.05) (Figure 37B) when compared to untreated cells. This represents internalization of the Dectin-1/glucan complex. Pre-treatment with hyperosmotic sucrose alone did not significantly affect the amount of Dectin-1 on the cell surface when compared to control cells (p>0.05) (Figure 37A). Furthermore, incubation of the sucrose treated cells with glucan did
Figure 37  Internalization of the Dectin-1/glucan complex is mediated by clathrin. Thioglycollate elicited macrophages were incubated with media alone or fluorescent labeled GP (10 µg/ml) for 3 h. Treated cells were pre-incubated with MβCD (5 mM) or sucrose (500 mM) for one hour. Cells were stained with anti-Dectin antibody and analyzed by flow cytometry for Dectin-1 expression (A.) and glucan uptake (B.). Data represent the mean of 4 independent experiments with n=3. * indicates p<0.05 compared to untreated control and + indicates p<0.05 compared to untreated glucan.

not change the amount of Dectin-1 on the cell surface while increasing the glucan fluorescence by 775% when compared to controls (p<0.05) (Figures 37A and B, respectively). This indicates that the sucrose is inhibiting Dectin-1/glucan internalization suggesting that glucan is being internalized by a clathrin mediated mechanism. In contrast, MβCD treatment alone decreased Dectin-1 surface expression by 31.75% when compared to untreated cells (p<0.05) (Figure 37A). Glucan treated MβCD cells had a 37.27% decrease in the percentage of Dectin-1 positive cells when compared to MβCD alone, and a 57.18% decrease when compared to untreated control cells (p<0.05) (Figure 37A). Interestingly, the MβCD treated cells internalized twice as much glucan than untreated cells with a 56.35% increase in the mean glucan fluorescence when compared to glucan treatment alone (p<0.05) (Figure 37B).
To confirm these data and to explain the slight increase in glucan fluorescence with hypertonic sucrose, macrophages were pre-incubated with hypertonic sucrose, MβCD, or media alone followed by incubation with fluorescent labeled glucan (green, Figure 38) for 6 h. The lipid rafts were stained (red, Figure 38) and the cells were imaged by confocal microscopy. The images confirm that hypertonic sucrose decreases the uptake of glucan by macrophages (Figure 38C) when compared to the untreated cells (Figure 38A). Additionally, the majority of glucan present with sucrose treatment was found extracellularly (Figure 38C). MβCD treatment was again found to increase glucan uptake (Figure 38B) when compared to the untreated image (Figure 38A). These data confirm that glucan internalization is clathrin dependent; however, lipid rafts appear to play a role in the regulation of internalization of glucans by macrophages.
Figure 38  GP internalization is mediated by clathrin Thioglycollate elicited macrophages were incubated with media alone or fluorescent labeled GP (green, 100 µg/ml) for 6 h. Treated cells were pre-incubated with media alone (A), MβCD (B), or hypertonic sucrose (C) for 1 hour. Cells were fluorescently stained for lipid rafts (red) and imaged by confocal microscopy. Representative images.

To further elucidate the role of lipid rafts and caveolin in the internalization of Dectin-1/glucan complexes, macrophages from caveolin-1 knock-out (Cav-/-) and wild type (WT) mice were incubated with fluorescent labeled glucan, stained for Dectin-1 expression, and analyzed by flow cytometry. The percentage of Dectin-1 expressing Cav-/- macrophages was significantly less than that of WT (89.65% vs. 62.73%, respectively) (Figure 39A). Though there was no difference in the decrease in the percentage of Dectin-1 positive cells with glucan treatment between Cav-/- and WT cells (12.49% vs. 12.79%, respectively) (Figure 39A), the gluca mean fluorescence of Cav-/- cells was 90.95% higher than WT cells (Figure 39B). These data again
suggest a regulatory role for lipid rafts and caveolin-1 in the internalization of Dectin-1/glucan complexes, and demonstrate that internalization is not mediated by lipid rafts or caveolin-1.

Figure 39  Internalization of the Dectin-1/glucan complex is enhanced in macrophages from caveolin-1 knock-out mice. Thioglycollate elicited macrophages from caveolin-1 knockout (Cav-/-) and wild type (WT) mice were incubated with fluorescent labeled glucan for 3 h. The cells were stained with anti-Dectin-1 antibody and analyzed for Dectin-1 expression (A) and glucan uptake (B) by flow cytometry. Average of 3 independent experiments with n=3, values are means ± SEM. * indicates p<0.05 compared to WT control, + indicates p<0.05 compared to WT glucan.

GP is not Trafficked to Lysosomes in Macrophages

The previous data have demonstrated that GP is internalized by a clathrin mediated mechanism; however, it is not known how the GP is trafficked after it has been internalized. Conventional wisdom implies that GP is trafficked via endosomes and to lysosomes (McCann et al. 2005). To determine if this is the case, thioglycollate elicited macrophages were incubated with fluorescent labeled glucan (green, Figures 40A and 41A and E). The cells were fixed, permeablized, and stained with antibodies against lysosomes (lamp1) (red, Figure 40B) or
endosomes (rab5b) (red, Figures 41B and F). Analysis by confocal microscopy revealed that GP does not co-localize with lysosomes (yellow and white, Figures 40C and D, respectively). Less than 20% of cells were positive for co-localization of GP with lamp-1 at any time interval tested.

Figure 40  GP is not trafficked to Lamp1 positive vesicles (lysosomes) in macrophages. Thioglycollate elicited murine macrophages were incubated with fluorescently labeled glucan phosphate (green, A) for 30 m and then stained with labeled anti-Lamp1 (red, B) antibody. Co-localization is shown in yellow in the overlay image (C) and in white in the masked image (D). A mask intensity rate ≥ 35% was considered positive. Representative images.

However, confocal analysis did not conclusively determine if GP was trafficked via endosomes as co-localization with endosomes was quite variable from experiment to experiment. At the 30 m incubation time, for example, one experiment found 0% of the cells had GP co-localized with rab5 (yellow and white, Figure 41C and D, respectively), but in another 100% of the cells had GP co-localized with endosomes (yellow and white, Figures 41G and H, respectively. These data suggest that GP can be both trafficked via endosomes and via an alternate pathway; perhaps caveosomes.
Figure 41  GP is trafficked by rab5B positive vesicles (endosomes) and by another type of vesicle in macrophages. Thioglycollate elicited murine macrophages were incubated with fluorescently labeled glucan phosphate (green, A and E) for 30 m and then stained with labeled anti-rab5B antibody (red, B and F). Co-localization is shown in yellow in the overlay image (C and G) and in white in the masked image (D and H). A mask intensity rate \( \geq 35\% \) was considered positive. Representative images.

GP is Trafficked to the Golgi Apparatus in Macrophages

As the lysosome has been ruled out as a destination for internalized glucan, alternate organelles were explored. Primary macrophages were incubated with labeled glucan (green, Figure 42A) and then stained for the Golgi apparatus (GM130) (red, Figure 42B), endoplasmic reticulum (GRP78) (data not shown), and the mitochondria (data not shown). Confocal analysis revealed that GP did not co-localize with either the endoplasmic reticulum or the mitochondria (data not shown). However, GP did co-localize with the Golgi apparatus (yellow, Figure 42C), with 100% of cells having a mask intensity rate greater than 35% at the 1 h incubation time (white, Figure 42D). Additionally, in 100% of cells GP was found to co-localize with the Golgi
apparatus at 30 m. These data indicate that GP is internalized by clathrin and trafficked by endosomes and other vesicles to the Golgi apparatus.

![Figure 42](image)

**Figure 42** GP is trafficked to the Golgi apparatus in macrophages. Thioglycollate elicited murine macrophages were incubated with fluorescently labeled glucan phosphate (green, A) for 1 h and then stained with labeled anti-GM130 antibody (red, B). Co-localization is shown in yellow in the overlay image (C) and in white in the masked image (D). A mask intensity rate \( \geq 35\% \) was considered positive. Representative images.

GP Co-Localizes with the Signaling Components P85, SR-A, and TLR2 in Macrophages

Previous studies have revealed that GP activates the PI3K signaling pathway (Williams et al. 2004a). Additionally, GP has been found to bind to scavenger receptor A (SR-A) (Rice et al. 2002). Finally, Dectin-1 signaling has been found to occur by both TLR2 dependent and independent pathways (Brown et al. 2003; Gantner et al. 2003). To determine if the Dectin-1/GP complex directly interacts with these signaling components, elicited macrophages were incubated with fluorescent labeled GP (green, Figures 43, 44, and 45A), fixed, and permeablized, and stained for the regulatory subunit of PI3K (p85) (red, Figure 43B), SR-A, (red, Figure 44B) and TLR2 (red, Figure 45B). P85 was found to consistently co-localize with GP in the majority of cells at time intervals from 5 m to 1 h (yellow, Figure 43C). At 5 m GP was co-localized with p85 in 100% of GP containing cells (white, Figure 43D). The same was true for the 15, 30 m, and 1 h incubations (data not shown). This implies that p85 is present on the membrane of the GP containing trafficking vesicle as well as on the membrane of the Golgi apparatus.
A.

B.

C.

D.

Figure 43  GP co-localizes with the p85 regulatory subunit of PI3K in macrophages
Thioglycollate elicited murine macrophages were incubated with fluorescently labeled glucan phosphate (green, A) for 30 m and then stained with labeled anti-p85 antibody (red, B). Co-localization is shown in yellow in the overlay image (C) and in white in the masked image (D). A mask intensity rate $\geq 35\%$ was considered positive for co-localization. Representative image.

GP was also found to co-localize with SR-A at time intervals from 5 m to 30 m, with 100% of GP containing cells being positive for co-localization with SR-A at 5, 15, 30 m, and 1 h (yellow or white, Figure 44C and D, respectively, and data not shown). This suggests that SR-A is being internalized with the Dectin-1/glucan complex and is trafficked with GP inside the cell.

A.

B.

C.

D.

Figure 44  GP co-localizes with scavenger receptor A after uptake by macrophages
Thioglycollate elicited murine macrophages were incubated with fluorescently labeled glucan phosphate (green, A) for 15 m and then stained with labeled anti-SRA antibody (red, B). Co-localization is shown in yellow in the overlay image (C) and in white in the masked image (D). A mask intensity rate $\geq 35\%$ was considered positive.

Finally, confocal analysis revealed that GP co-localized with TLR2 in 100% of GP containing cells from 5 m to 1 h (yellow or white, Figure 45C and D, respectively, and data not
shown). These data imply that TLR2 is also internalized with the Dectin-1/glucan complex and trafficked within the cell with GP.

Figure 45  GP co-localizes with Toll-Like Receptor 2 at 1 hour after internalization by macrophages. Thioglycollate elicited murine macrophages were incubated with fluorescently labeled glucan phosphate (green, A) for 1h and then stained with labeled anti-TLR2 antibody (red, B). Co-localization is shown in yellow in the overlay image (C) and in white in the masked image (D). A mask intensity rate ≥ 35% was considered positive for co-localization. Representative image.

Different Types of Glucans are Similarly Trafficked by Macrophages

As previously mentioned, different glucans have different activities in vivo. For example, GP is protective in sepsis and causes a prolonged decrease in Dectin-1 surface expression on blood neutrophils. Laminarin, a soluble glucan that is derived from *Laminaria digitata*, does not protect in sepsis and only transiently decreases Dectin-1 on neutrophils. It is possible that the differences in activity, especially in regards to Dectin-1 surface expression, may be because of differences in trafficking. In fact, Herre et al. have reported that upon internalization with GP Dectin-1 is degraded, but that when internalized with laminarin it is recycled back to the cell surface. To determine if laminarin is trafficked differently from GP, fluorescent labeled laminarin (green, Figures 46A, E, I, M, and Q) was incubated with thioglycollate elicited macrophages, and then the cells were stained for clathrin (heavy chain), caveolin-1, endosomes (rab5), Golgi apparatus (GM130), or lysosomes (lamp1) (red, Figure 46B, F, J, N, and T, 103
respectively). Confocal microscopy revealed that, unlike GP, laminarin does not co-localize with clathrin (yellow and white, Figures 46C and D, respectively); however, it does co-localize with caveolin-1 (yellow and white, Figures 46G and H, respectively). Mask intensity analysis revealed that in 60.87% of cells laminarin co-localizes with caveolin-1 at 30 m and 62.50% are co-localized at 1 h. This suggests that laminarin is internalized by a lipid raft/caveolin mediated mechanism rather than clathrin mediated. Unlike GP, laminarin does not co-localize with endosomes at any time interval tested (yellow and white, Figure 46K and L, respectively). Like GP, laminarin does not co-localize lysosomes (yellow and white, Figures 46S and T, respectively), but, though it co-localize with the Golgi apparatus, co-localization is only seen in 52.90% of cells at the 30 m incubation (data not shown) while 0% of cells had laminarin co-localized with the Golgi at 1 h (yellow and white, Figure 46O and P, respectively). These data suggest that intracellular trafficking of glucans is dependent upon their structure. As laminarin is found to co-localize with caveolin-1 and only transiently with the Golgi apparatus, it is possible that the Dectin-1 and laminarin are being rapidly transported back to the cell surface. This would explain the transient effect of laminarin on Dectin-1 expression on the cell surface.
Figure 46 Laminarin is internalized by Caveolin-1 positive vesicles. Thioglycollate elicited murine macrophages were incubated with fluorescently labeled laminarin (green, first column), and then stained with labeled antibodies against intracellular organelles (red, second column). Co-localization of the laminarin with the organelle is indicated in the overlay image by yellow (third column) and by white in the masked image (fourth column). A mask intensity rate ≥ 35% was considered positive for co-localization.

Particulate and Soluble GPs are Similarly Trafficked by Macrophages

The GP and laminarin used in the previous studies are soluble glucans. To determine if trafficking of particulate glucans is similar to that of soluble glucans, thioglycollate elicited macrophages were incubated with fluorescent labeled particulate GP (green, Figures 47A, E, I, M, and Q). Particulate GP is similar in activity to the soluble product in that it is protective in
polymicrobial sepsis (Williams et al. 1980). The cells were fixed, permeablized, and stained with antibodies against clathrin (heavy chain), caveolin-1, endosomes (rab5), Golgi apparatus (GM130), and lysosomes (lamp1) (red, Figures 47B, F, J, N, and R, respectively). Based on the fluorescent properties of the particulate ligand and a distribution analysis, the mask intensity rate for co-localization with the particulates was reduced to 20%. Analysis revealed that only 23.53% of cells were positive for co-localization between particulate GP and clathrin (yellow and white, Figures 47C and D, respectively). However, 100% of cells were positive for co-localization between particulate GP and caveolin-1 (yellow and white, Figures 47G and H, respectively). Co-localization with endosomes was clearly evident in the case of the particulate GP with 85.71% of cells being positive (yellow and white, Figures 47K and L, respectively). Particulate GP also co-localizes with the Golgi apparatus at all time intervals tested with a mean of 68.56% of cells analyzed being positive for co-localization (yellow and white, Figures 47O and P, respectively). Like other glucan ligands, particulate GP does not co-localize with lysosomes (yellow and white, Figures S and T, respectively) with a mean of 36.25% of cells found to have a mask intensity rate greater than 20%. These data imply that particulate GP is internalized into a caveosome and trafficked by endosomes to the Golgi apparatus.
Figure 47 Particulate glucan is internalized by Caveolin-1 and Rab5B positive vesicles. It is trafficked to the Golgi but not to lysosomes. Thioglycollate elicited murine macrophages were incubated with fluorescently labeled particulate glucan (green, first column) and then stained with labeled antibodies against intracellular organelles (red, second column). Co-localization of the glucan with the organelle is indicated in the overlay image by yellow (third column) and by white in the masked image (fourth column). A mask intensity rate $\geq 20\%$ was considered positive for co-localization.
Previous studies have found that Dectin-1 is the primary PRR for \((1\rightarrow3)-(1\rightarrow6)-\beta-D\)-glucans (Brown and Gordon 2001; Willment et al. 2001; Brown et al. 2002; Taylor et al. 2002; Brown et al. 2003). Brown et al. demonstrated that Dectin-1 mediates the immune response to fungal glucans, and Taylor et al. found that Dectin-1 is present on innate immune cells such as neutrophils, dendritic cells, and macrophages (Brown and Gordon 2001; Willment et al. 2001; Brown et al. 2002; Taylor et al. 2002; Brown et al. 2003). Brown et al. suggested that Dectin-1 is a sentinel receptor for fungal infection (Brown et al. 2003). Though these studies have provided a wealth of important information regarding Dectin-1 and glucan, they were performed using cell culture or soluble Dectin-1. No prior studies have addressed Dectin-1/glucan interactions \textit{in vivo}. Furthermore, the role of Dectin-1 in polymicrobial sepsis has not been investigated even though the ligand for Dectin-1 improves survival in polymicrobial sepsis. The present work revealed the effects of fungal infection, polymicrobial sepsis, and glucan administration on Dectin-1 \textit{in vivo} (Table 2).
Table 2  Summary of the effects of fungal infection, polymicrobial sepsis, and glucan administration on Dectin-1 expression in the blood

<table>
<thead>
<tr>
<th>Study Condition</th>
<th>Dectin Positive Leukocytes</th>
<th>Dectin Positive Neutrophils</th>
<th>Dectin Positive Monocytes</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. albicans infection</td>
<td>Increased</td>
<td>No Change</td>
<td>No Change</td>
<td>Neutrophilia, Neutrophils with less Dectin-1</td>
</tr>
<tr>
<td>Polymicrobial Sepsis</td>
<td>Decreased</td>
<td>Decreased</td>
<td>No Change</td>
<td>------</td>
</tr>
<tr>
<td>Glucan administration</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Decreased</td>
<td>Decreased Dectin-1 Positive Monocytes</td>
</tr>
</tbody>
</table>

Dectin-1 Expression is Increased by *C. albicans* Infection

In Specific Aim 1 we examined the effect of systemic *C. albicans* infection on Dectin-1. We found that following *C. albicans* infection there was an overall increase in Dectin-1 levels in the blood, spleen, and kidneys of infected mice. The change in overall Dectin-1 levels in the blood and spleen were primarily because of an influx of Dectin-1 positive neutrophils, though the amount of Dectin-1 expressed by these cells was reduced. In the kidney, Dectin-1 positive macrophages and neutrophils were found at foci of fungal infection. We also observed that the overall percentage of T cells in the periphery and spleen was decreased in the response to candidiasis. However, the remaining T cell populations showed a higher percentage of Dectin-1 expressing cells when compared to control. These data demonstrate that Dectin-1 expression is altered by a clinically relevant fungal infection.
C. albicans infection induced a Dectin-1 positive neutrophilia and an infiltration of the kidneys with Dectin-1 expressing cells. Neutrophils are the first line of defense against fungal infections (Romani 2004), thus it is not surprising that candidiasis induced neutrophilia. Dectin-1 is expressed at high levels on neutrophils (Brown et al. 2002; Taylor et al. 2002). Therefore, a Dectin-1 positive neutrophilia may be interpreted as an innate immune response to candidal infection. However, the increase in Dectin-1 positive cells was not sufficient to clear the fungal pathogen as C. albicans continued to be cultured from kidneys at the onset of mortality. The increase in Dectin-1 was also not sufficient to alter disease outcome because the mortality rate was 100% by day 21 of infection. This may be because of the challenge dose of C. albicans that may have been of sufficient magnitude to overwhelm innate host defenses, leading to a productive infection and ultimately death of the host. There are other possible explanations for this outcome. Recent data from Netea and colleagues have demonstrated that TLR2 knockout mice, which were challenged with C. albicans, showed increased rates of survival when compared to wild type mice (Netea et al. 2004). The presence of TLR2 during candidiasis also resulted in increased production of IL-10, an anti-inflammatory cytokine that down regulates various aspects of immunity (Netea et al. 2004). This indicates that TLR2 is not protective; rather it contributes to the pathophysiology of candidiasis. Because Dectin-1 and TLR2 are known to collaborate in order to elicit an inflammatory response (Brown et al. 2003; Gantner et al. 2003), it is possible that Dectin-1, much like TLR2, may not play a protective role in response to C. albicans induced sepsis. An alternative explanation may involve the loss of Dectin-1 on neutrophils. Decreased neutrophil Dectin-1 levels may result in neutrophils that are less able to bind, internalize, and destroy fungal elements. Additionally, the Dectin-1 positive cells found in the C. albicans containing renal lesions were primarily macrophages. Macrophages are less
effective killers of *C. albicans* because the fungi are able to escape the phagolysosome and survive within the cell (Bodey 2000). If the neutrophils in the renal foci of infection have lost Dectin-1 on their cell surface, they would be less able to bind, internalize, and destroy the pathogen. This would allow for further growth of the organisms at each focus of infection. We have determined the effect of *C. albicans* on Dectin-1 *in vivo*; however, the precise role of Dectin-1 in response to fungal infection will only be determined when studies with Dectin-1 knockout mice are conducted.

**Dectin-1 Expression is Decreased by Polymicrobial Sepsis**

Specific Aim 2 determined the effect of a clinically relevant *in vivo* model of sepsis, with and without glucan administration, on cell surface Dectin-1 expression. We found that polymicrobial sepsis decreased the number of Dectin-1\textsuperscript{high} positive blood leukocytes while increasing the numbers of Dectin-1\textsuperscript{low} positive cells. This represents a shift from a high positive to a low positive phenotype by cells considered the first line of defense, neutrophils, and, to a lesser extent, monocytes. Additionally, glucan administration in sepsis caused a further decrease in blood leukocyte Dectin-1 expression as well as a decrease in splenic and peritoneal cell Dectin-1 expression. Again, a loss of cell surface Dectin-1 from neutrophils resulted in this decrease. Because glucan administration in sepsis decreases Dectin-1 expression and improves survival, a loss of Dectin-1 on leukocytes positively correlates with improved survival.

Glucan administration in polymicrobial sepsis decreased the percentage of Dectin-1 positive neutrophils and peritoneal cells. Though the decrease in Dectin-1 expression with glucan administration in sepsis is not surprising based on studies conducted with glucan administration *in vitro*, it is interesting to speculate how loss of cell surface Dectin-1 may
contribute to the improved survival found with glucan administration in sepsis. One possibility may relate to the study by Ariizumi et al. who demonstrated that “Dectin-1 proteins… bind to putative ligand(s) on T cells and deliver co-stimulatory signals” (Ariizumi et al. 2000). Thus, 1 way in which glucan may promote survival in sepsis is by preventing the interaction of Dectin-1 with its T cell ligand. Further supporting this notion, studies indicate that interaction between T lymphocytes and monocytes or neutrophils, cells that express high levels of Dectin-1, results in a pro-inflammatory state (Li et al. 1995; Burger and Dayer 2002a; Burger and Dayer 2002b). It is possible that the association of Dectin-1 with the T cell ligand is contributing to septic inflammation. Loss of Dectin-1 from the cell surface because of glucan administration might prevent the interaction of Dectin-1 with the T cell ligand, thus, tempering the overall inflammatory state, and, thereby, promoting survival. Identification of the T cell ligand for Dectin-1 and determination of the effect of the ligand’s interaction with Dectin-1 will provide further insight into the role of Dectin-1 in sepsis and possibly other inflammatory diseases as well.

We demonstrated that polymicrobial sepsis decreased the percentage of neutrophils that express Dectin-1. Because the effect of sepsis on Dectin-1 expression has not previously been investigated, the mechanisms that are responsible for the decrease in Dectin-1 expression have yet to be determined. One possible explanation for this effect would be elevated levels of circulating glucans. Digby et al. demonstrated that septic patients have an increase in circulating glucans regardless of infection type (Digby et al. 2003). It has been proposed that in the critically ill, glucans translocate from the gut into the systemic circulation (Rice et al. 2005b). In the present study, polymicrobial sepsis was induced by introduction of cecal contents into the abdominal cavity. Not only is it possible that glucans are translocating from the gut in this
model, but the ingesta from the cecum includes large numbers of microbes; therefore, the material extruded from the cecum into the peritoneal cavity contains glucan from the cell walls of these microbes. The glucan from the peritoneal cavity could be absorbed into the systemic circulation, bind to Dectin-1, and cause Dectin-1 internalization and cell signaling.

Alternatively, other factors have been shown to affect Dectin-1 expression (Willment et al. 2003). Willment et al. have reported that interleukin (IL)-10 and LPS negatively regulate Dectin-1 RNA expression in vitro (Willment et al. 2003). The CLP model of polymicrobial sepsis results in elevated levels of circulating LPS and cytokines. Studies have also shown that septic patients tend to have an increase in IL-10 (Marchant et al. 1994). Any or all of these factors might contribute to the decreased cell surface expression of Dectin-1 that was observed; therefore, in vitro studies were performed to elucidate the mechanisms of decreased cell surface expression of Dectin-1.

The in vivo studies demonstrated a differential effect on Dectin-1 expression with C. albicans infection and polymicrobial sepsis. In polymicrobial sepsis there is a profound decrease in the percentage of neutrophils that express Dectin-1. In C. albicans infection the percentage of Dectin-1 positive neutrophils remained unchanged, but the amount of Dectin-1 expressed by neutrophils was moderately decreased. The in vitro studies were performed to determine the mechanism responsible for the observed differences. We found that glucan administration alone resulted in loss of Dectin-1 from the neutrophil cell surface. Non-glucan PAMPs such as LPS and mannan did not alter neutrophil Dectin-1 unless the cells were also treated with glucan. This suggests that glucans are the only PAMPs that affect Dectin-1 surface expression on neutrophils. Though it seems counter-intuitive, the in vivo and in vitro data together suggest that the levels of circulating glucans may be higher in mice with polymicrobial sepsis than in those with fungal
sepsis. In fact, clinical studies by Digby et al. demonstrated that levels of circulating glucans in the critically ill were independent of the presence or type of infection (Digby et al. 2003). These results may have clinical relevance. It has been proposed that monitoring increases in serum glucan levels may be useful as a diagnostic assay for fungal infections (Kohno et al. 1993; Mori et al. 1997; Yoshida et al. 1997). The FDA has recently approved a glucan specific serum assay as an indicator of fungal sepsis (Odavasi et al. 2004). However, the present data as well as the previous clinical studies (Digby et al. 2003) suggest that measuring serum glucan levels as a positive predictive indicator for fungal infections is imprudent.

Dectin-1 Expression is Decreased by Administration of Glucans

Specific Aim 3 evaluated the effect of glucan administration on Dectin-1 expression. Previous studies have demonstrated that glucans are rapidly internalized by macrophage and monocyte cell lines, and that Dectin-1 is internalized in response to glucan treatment in cultured macrophages (Mueller et al. 1996; Herre et al. 2004). We found that systemically administered glucans are uptaken from the circulation by peripheral leukocytes, and the glucan-Dectin-1 complex is internalized and co-localized within the leukocyte. Thus, we conclude that the loss of Dectin-1 from the leukocyte membrane is because of internalization of the glucan-Dectin-1 complex, following receptor ligand interaction. We also observed that a single injection of GP resulted in decreased peripheral leukocyte Dectin-1 positivity for up to 7 days. Splenic and peritoneal leukocytes also showed a decrement in surface Dectin-1 following glucan administration, but the magnitude and duration was less than that observed in peripheral leukocytes. While Dectin-1 levels were significantly decreased on circulating leukocytes in response to glucan, we did not observe a complete loss of leukocyte membrane Dectin-1. The
maximum decrement was approximately 85%, even in the presence of a high loading dose of GP. The loss of Dectin from the cell membrane was primarily observed in peripheral neutrophils and monocytes, though no dramatic changes were found in overall leukocyte numbers. Therefore, systemic administration of glucan results in a preferential decrease in neutrophil and monocyte Dectin-1 levels because of internalization of the glucan-Dectin complex but does not change the peripheral leukocyte levels.

We determined that administration of GP reduced Dectin-1 expression on blood neutrophils and monocytes for up to 7 days. The prolonged duration of effect of GP on Dectin-1 levels was an unexpected finding for several reasons. First, the effect of glucan on Dectin-1 was primarily observed in neutrophils and monocytes. These leukocytes are present in the systemic circulation for relatively short time periods, i.e. hours or days. Second, Rice et al. have reported that GP has a distribution half-life of 4.3 ± 0.7 min, an elimination half-life of 3.8 ± 0.8 hrs, a volume of distribution of 350 ± 88 ml/kg and a clearance rate of 42 ± 6 ml/kg hr from the plasma following IV administration (Rice et al. 2004). Thus, the effect of glucan on leukocyte membrane Dectin-1 levels lasts beyond the expected half-life of neutrophils and monocytes and exceeds the anticipated clearance of the glucan from the systemic circulation. As glucans have been reported to modulate leukocyte bone marrow precursors (Patchen and Lotzova 1980), 1 possible explanation for the prolonged effect was that glucan administration altered Dectin-1 membrane expression at the level of monocyte and/or neutrophil precursors in the bone marrow. However, we found that glucan did not alter Dectin-1 levels in bone marrow cells. Another potential explanation for the prolonged effect of glucan administration on Dectin-1 cell surface expression may be recycling of the glucan. Recent studies by Hong et al. suggest that orally administered water insoluble glucans are uptaken by macrophages and transported to the spleen,
lymph nodes, and bone marrow (Hong et al. 2004). The authors speculated that the glucan was degraded and released into the systemic circulation where it was subsequently internalized by granulocytes (Hong et al. 2004). However, mammalian cells do not have the enzymes necessary to specifically catabolize glucans (Stone and Clark 1992); therefore, a more reasonable explanation is that leukocytes which have bound and internalized glucan may release the glucan at the end of their lifecycle and it is then internalized by leukocytes which have recently entered the systemic circulation. In support of this concept, Monari et al. have reported that neutrophils rapidly ingest glucuronoxylomannan and then subsequently released the carbohydrate (Monari et al. 2003). This effect may not be limited to leukocytes. Receptors for glucans have been identified on vascular endothelial cells, epithelial cells, and fibroblasts (Ahren et al. 2001; Kougias et al. 2001; Lowe et al. 2002). Thus, it is possible that glucans are uptaken from the systemic circulation by a variety of cells and tissues and are then slowly released over time to be internalized by newly released circulating leukocytes. We also noted that membrane Dectin-1 levels normalized by day 7 post-injection. We know that Dectin-1 receptors do not recycle to the cell surface once they are complexed and internalized with a biologically active glucan such as glucan phosphate (Herre et al. 2004). New receptors must be synthesized and expressed (Herre et al. 2004). Thus, the re-appearance of leukocyte membrane Dectin-1 in our study may relate to prolonged elimination of the glucan from the body as well as synthesis of new receptor in some leukocytes. Further studies will be necessary to determine which mechanism(s) are primarily involved in the prolonged response to parenterally administered soluble glucans.

In an attempt to better understand the role of Dectin-1 in the biologic activity of glucans, we compared and contrasted the effect of an inactive glucan, laminarin, on leukocyte membrane associated leukocyte Dectin-1 levels. Laminarin was studied as it has been used for *in vitro*
studies as a Dectin-1 antagonist (Brown et al. 2002; Gantner et al. 2003). Additionally, we have found that laminarin is not protective in sepsis; in fact, it actually shortens the time to mortality. Administration of laminarin reduced leukocyte cell surface Dectin-1, but the effect was quite transient when compared to GP. The reason for the difference in effect on Dectin-1 expression is possibly related to the intracellular trafficking of Dectin-1 upon internalization with glucans. Previous studies have found that when Dectin-1 is internalized with laminarin, it is recycled to the cell surface (Herre et al. 2004). When Dectin-1 binds and internalizes GP, the receptor does not recycle to the cell surface (Herre et al. 2004). New receptor must be synthesized and expressed on the cell surface (Herre et al. 2004). Interestingly, the duration of the effect of laminarin on Dectin-1 expression is similar to the circulating lifespan of neutrophils and monocytes. It is reasonable to speculate that laminarin’s activity is limited only to the cells circulating at the time of administration. Regardless of the underlying mechanism, the differential effect of laminarin, which does not protect in sepsis, and GP, which is protective in sepsis, on Dectin-1 expression further supports the notion that Dectin-1 plays a significant role in the protection conferred by Dectin-1 in sepsis.

To determine the specificity of the effect of glucans on Dectin-1 expression in vivo, we compared and contrasted the effect of non-glucan carbohydrate polymers on leukocyte membrane associated leukocyte Dectin-1 levels. We employed mannan and pullulan as control polymers for several reasons. First, they are non-β linked carbohydrate polymers and they are not ligands for Dectin-1 (Brown et al. 2002). In addition, mannan is a mannose polymer that is found in association with glucan in the fungal cell wall (Stone and Clark 1992). The mannan employed in this study was isolated from the same fungal source as the GP (Peat et al. 1961). While pullulan had no effect on membrane Dectin-1 levels, we observed a differential effect of
GP and mannan on leukocyte Dectin-1 positivity. While GP resulted in an overall loss of leukocyte Dectin-1 positivity, mannan resulted in a modest, but significant, increase in leukocyte Dectin-1 levels. Thus, the loss of leukocyte membrane associated Dectin-1 is specific for glucan polymers and not a non-specific effect of carbohydrate polymers in general. It is interesting to note that mannan also results in a neutrophilic leukocytosis. This is similar to the effect of *C. albicans* infection. It is highly possible that mannan from the *C. albicans* cell wall is responsible for the change in blood leukocyte population dynamics seen with *C. albicans* infection.

The role of internalization of the Dectin-1/glucan complex in the biologic activities is controversial. Numerous studies have shown that systemic administration of pharmaceutical grade glucans will stimulate innate immunity (Williams et al. 1999b), increase resistance to infectious challenge (Williams et al. 1978; Williams et al. 1982; Williams et al. 1988; Williams et al. 1991b), decrease myocardial injury following ischemia/reperfusion injury (Li et al. 2003), suppress the growth of transplanted tumors (Cheung et al. 2002; Hong et al. 2004), and facilitate wound repair (Wei et al. 2002). The cellular and molecular mechanisms by which glucans mediate these effects are currently the subject of intense investigation (Williams et al. 1999b; Williams et al. 2004b). Several investigators speculate that Dectin-1 mediates the biological effects of glucans (Brown et al. 2003; Gantner et al. 2003). Indeed, it has been reported that ligation of Dectin-1 is sufficient to transduce an activating signal into macrophages and that internalization of the ligand-receptor complex is not required for induction of biological activity (Brown et al. 2003; McCann et al. 2005). However, those experiments were performed with cultured cells, the stimulus was zymosan, a glucan-mannan cell wall extract, and the endpoint was cytokine production, not modification of disease (Brown et al. 2003). In addition, we have shown that a single injection of glucan, either IV or IP, can induce biological effects lasting up to
7 days (Williams et al. 1999b). This coincides with the period when leukocyte Dectin-1 levels are decreased. This may indicate that binding and internalization of glucans by Dectin-1 are crucial to expression of in vivo biological activity. As the Dectin-1 response to both IV and IP administration of GP are not significantly different at most time points, these data also support previous findings that both the IV and IP routes of administration are equally effective in modulating immune responses (Williams et al. 1999b). These data may have significant implications for glucan dosing regimens.

Modulation of Cytokines and Chemokines by C. albicans, Polymicrobial Sepsis, and Glucan Administration

Cytokines and chemokines are integral to an appropriately functioning immune response. In the case of C. albicans infection, genetic depletion of TNFα, IL-6, IL-17, or IFNγ impairs the immune response to C. albicans and diminishes survival (Romani et al. 1996; Mencacci et al. 1998; Balish et al. 1998). An overproduction of inflammatory cytokines, including TNFα, IL-1β, and IL-6, is thought to be a key mediator of sepsis syndrome (Damas et al. 1992; Pinsky et al. 1993). Despite the fact that glucans have been found to stimulate the production of TNFα in vitro (Brown et al. 2003; Gantner et al. 2003), there are no data regarding the cytokine and chemokine profile of animals treated with glucans. Furthermore, cytokines have been found to modulate Dectin-1 expression (Willment et al. 2003). While cytokines and chemokines have been studied in C. albicans and in sepsis, only a few have been measured at a time because of technological constraints. With the development of multiplex technology we were able to simultaneously measure up to 16 cytokines and chemokines in 1 sample. This approach allowed
us to make the following new and novel observations regarding cytokine and chemokine
dynamics in our in vivo models.

**The Cytokine and Chemokine Profile of Mice Infected with *C. albicans***

We found that in *C. albicans* infection the inflammatory cytokines TNFα and IL-6 as well as the neutrophil chemokine IL-17 were elevated late in the course of disease, while the anti-inflammatory cytokine, IL-10, was decreased. This inflammatory profile coincided with the onset of mortality. Although these changes have been associated with candidiasis in the past (Steinshamn and Waage 1992; Huang et al. 2004), this is the first report of this particular temporal response. This may indicate that an overall inflammatory state is contributing to mortality in this model, as previous studies have shown that inflammatory cytokines, such as TNFα and IL-6, are associated with morbidity and mortality in response to infectious disease (Tracey et al. 1986; Bauss et al. 1987).

IFNγ is thought to play an important role in innate host defense against *C. albicans* infection (Kullberg et al. 1993; Balish et al. 1998). We were unable to detect a significant change in serum levels of IFNγ, although there was a trend towards increased levels on day 1. Interestingly, the IFNγ induced chemokine MIG was significantly increased in *C. albicans* challenged mice on day 1. MIG is a chemoattractant for activated lymphocytes that is produced by antigen presenting cells (Park et al. 2002). MIG is involved in recruitment of effector T cells to sites of peripheral cell mediated inflammation, notably in graft and tumor rejection (Tannenbaum et al. 1998; Koga et al. 1999; Miura et al. 2001). To the best of our knowledge, this is the first report of increased MIG levels in candidiasis. In this case, MIG may be involved
in initial recruitment of activated T lymphocytes to peripheral foci of *C. albicans* infection. This may account for the decrease in peripheral T cells observed during candidiasis.

**The Cytokine Profile of Mice with Polymicrobial Sepsis**

We found that CLP increased circulating levels of the pro-inflammatory cytokines TNFα, IL-6, and IL-1β. Levels of the anti-inflammatory cytokine IL-10 were also increased. This mixed inflammatory response is consistent with previous studies measuring cytokines in patients with sepsis (Damas et al. 1992; Pinsky et al. 1993; Marchant et al. 1994). Because SIRS has been associated with elevations in pro-inflammatory cytokines (Brunn and Platt 2006), and GP improves survival in sepsis by tempering the NF-κB signaling pathway (Williams et al. 1999b), one would expect that GP administration would reduce pro-inflammatory cytokines to near normal levels. However, this was not the case. GP administration in sepsis had no significant effect on circulating cytokines when compared to CLP alone. These data in combination with data showing that blocking inflammatory cytokine activity does not improve survival (Opal et al. 1997; Clark et al. 1998) would seem to further reduce the importance of cytokines in the pathophysiology of sepsis.

**Cytokine and Chemokine Profile of Glucan Treated Mice**

Prior to the present study, pure GP was not thought to alter cytokine or chemokine production. However, we have demonstrated that GP does alter cytokines and chemokines when administered parenterally. Administration of a single injection of GP increased the circulating concentrations of the neutrophil chemokines KC and IL-17 and the monocyte chemokine MCP-
1. GP also increased in the IFNγ dependent chemokines IP-10 and MIG and decreased the concentration of the Th1 regulatory cytokine IL-12, though it had no effect on the concentrations of IFNγ itself. No changes were found in the concentrations of TNFα, IL-1β, or IL-6, the traditional pro-inflammatory cytokines. Neither were changes found in the anti-inflammatory cytokine IL-10.

Administration of GP induced the production of neutrophil chemokines 24 hours after injection. Previous studies indicate that glucans stimulate neutrophil activity (Williams et al. 1988; Tsikitis et al. 2004; Sato et al. 2006); however, the effect of glucans on neutrophils was assumed to be direct, i.e. by binding of glucan to Dectin-1 and other glucan receptor(s) that are present on neutrophils (Taylor et al. 2002). Direct interaction may be one mechanism for the activation of neutrophils by glucans, but our data suggest an indirect activation of neutrophils by chemokines may occur as well. Glucan administration stimulated an increase in the neutrophil stimulants KC and IL-17. IL-17 is produced by T lymphocytes and is thought to be a link between the innate and adaptive immune response (Kawaguchi et al. 2004). IL-17 stimulates the production of neutrophils by increasing the concentration of G-CSF (Kawaguchi et al. 2004). As previously mentioned, IL-17 is necessary for the host immune response to C. albicans infection (Huang et al. 2004). IL-17 may also be involved in glucan mediated protection in sepsis. Rice et al. have demonstrated that administration of CpG DNA, a TLR9 ligand, is protective in CLP induced polymicrobial sepsis via an IL-17 dependent mechanism (Rice et al. 2005a). It is possible that the same is true for glucan mediated protection. Though IL-17’s effects are primarily on neutrophils, the IL-17 receptor is present on many different cell types (Kolls and Linden, 2004). IL-17 induces production of KC by monocytes and macrophages and induces IP-10 production by endothelial cells (Kawaguchi et al. 2004; Kolls and Linden, 2004). Thus, the
increase in IL-17 concentration may also result in the changes in the levels IP-10 and KC found with GP administration. These data suggest an alternative mechanism for activation of neutrophils by glucans.

GP administration increased the T lymphocyte chemokines IP-10 and MIG. These 2 chemokines share a receptor and have similar effects (Farber 1997). Both chemokines are produced by monocytes and macrophages and recruit activated T cells to the sites of production (Farber 1997). Synthesis of both chemokines is stimulated by IFNγ, though other mediators will also induce their production (Farber 1997; Kolls and Linden, 2004). Finally, both chemokines promote the generation of cell mediated immune responses and inhibit the humoral immune response (Farber 1997). The effect of increased IP-10 and MIG levels with GP administration is unclear; however, an increase in the concentration of T lymphocyte chemokines by GP further supports the argument that the glucan specific receptor, Dectin-1, is a link between innate and adaptive immunity.

By comparing the cytokine profile of mice injected with pure glucan with that of mice infected with whole *C. albicans*, we were able to isolate the effects of the fungal glucan on cytokine concentrations. Increased concentrations of MIG and IL-17 were detected with both *C. albicans* infection and GP injection; therefore, it is likely that it is the glucan in the *C. albicans* cell wall that is inducing the production of these cytokines. As GP did not induce production of the inflammatory cytokines TNFα or IL-6, these cytokines are being induced by another fungal PAMPs in the *C. albicans* cell wall.
Clathrin Mediated, Caveolin Regulated Internalization of Glucans

Specific Aim 4 examined intracellular trafficking of glucans. Previously it was assumed that glucans were internalized into endosomes and trafficked into lysosomes. Recent studies by McCann et al. determined that glucans are internalized by a non-lipid raft mediated mechanism (McCann et al. 2005). They also concluded that glucans were trafficked to lysosomes (McCann et al. 2005). This study investigated only particulate glucans. Additionally, regular fluorescent microscopy was used to determine co-localization (McCann et al. 2005). We sought to gain additional insights into the trafficking of glucans by evaluating three different glucans using confocal microscopy. We demonstrated that GP is internalized by a clathrin mediated mechanism that is regulated by caveolin-1 and/or lipid rafts (Figure 48). We found that GP is trafficked to the Golgi apparatus, not to the lysosomes as previously assumed (Figure 48). We also found that laminarin and particulate glucan are trafficked in a similar fashion. Not surprisingly, GP co-localizes with Dectin-1, but it also co-localizes with p85, SR-A, and TLR2 (Figure 48). These data support previous studies that indicate that these signaling components are involved in GP mediated signaling (Rice et al. 2002; Gantner et al. 2003; Williams et al. 2004a).
Figure 48  Schematic of glucan internalization and trafficking. Glucan is internalized with SR-A and TLR 2 by clathrin and trafficked via early endosomes to the Golgi apparatus. Internalization is negatively regulated by caveolin-1 by an unknown mechanism.

The evidence for internalization of the Dectin-1/GP complex by clathrin is clear cut; however, the role of lipid rafts and caveolin-1 in internalization of the complex is less clear. We have demonstrated that loss of caveolin-1 or lipid rafts results in enhanced internalization of GP despite a reduction in cell surface Dectin-1. It was found that GP co-localizes with caveolin-1. These data suggest that caveolin-1 and lipid rafts are regulating Dectin-1 cell surface expression and internalization. Caveolin-1 is known to interact with signaling molecules (Jayanthi et al. 2004; Williams and Lisanti 2004c). It is possible that Dectin-1 is normally sequestered into caveolae by caveolin-1, and internalization of GP by Dectin-1 is inhibited by this sequestration.
Loss of caveolae by disruption of lipid rafts or by genetic depletion of caveolin-1 relieves this inhibition allowing increased uptake of GP.

It is interesting to note that glucans are transported to the Golgi apparatus after internalization rather than to lysosomes. This is not an uncommon fate for PAMPs, as both LTA and LPS are transported to the Golgi apparatus after internalization (Thieblemont and Wright 1999; Latz et al. 2002; Triantafilou et al. 2004a), but it raises the question of what happens to the glucans once they have reached the Golgi. Mammalian cells do not possess the enzymes necessary for glucan catabolism (Stone and Clark 1992). Therefore, there are 2 possibilities for the fate of glucans after they reach the Golgi: the glucans may remain in the Golgi or another cellular compartment for the life of the cell or they may be transported out of the cell. If the macrophage excretes the GP over time this would explain the prolonged effect of GP on Dectin-1, as macrophages, and perhaps other cell types, could act as repositories for GP. Additionally, when a neutrophil undergoes apoptosis, the apoptotic bodies are frequently ingested by macrophages (Meszaros et al. 2000). This could lead to recycling of the GP contained within the neutrophil. Recycling of glucan by macrophages and other cell types would explain the prolonged effect of GP.

The trafficking studies demonstrate that laminarin and GP are trafficked in a similar fashion. These data do not explain the differences in the effect of the glucans on Dectin-1 expression, nor do they explain the work by Herre et al. that reports that Dectin-1 is degraded with GP but recycled with laminarin (Herre et al. 2004). One explanation for this discrepancy would be that Dectin-1 is dissociating from the glucan at some point after internalization. The Dectin-1 could then be processed differentially by the cell depending upon the ligand with which it was internalized. This speculation is supported by studies investigating the trafficking of
TLR4 and LPS (Triantafilou et al. 2002; Triantafilou et al. 2004a; Triantafilou et al. 2004b; Husebye et al. 2006). These studies demonstrated that TLR4 is trafficked to lysosomes where it is degraded, but the LPS is transported to the Golgi apparatus (Thieblemont and Wright 1999; Husebye et al. 2006). This implies that the TLR4/LPS complex is dissociated prior to transport of the individual molecules to their final destination (Husebye et al. 2006). Further studies will be necessary to determine the fate of Dectin-1 after internalization with glucan.

In conclusion, we have confirmed the hypotheses that cell surface Dectin-1 is altered by fungal infection, polymicrobial sepsis, and glucan administration. We have determined that glucan is the PAMP responsible for loss of Dectin-1 demonstrated in the *in vivo* models. Additionally, we have discovered the mechanisms of internalization and trafficking of glucans. These studies have provided the groundwork for a better understanding of the role of Dectin-1 and glucan in the innate immune response.
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APPENDIX

Abbreviations

AF - Alexa Fluor™
ANOVA - analysis of variance
BAP - blood agar plate
Cav-/- - caveolin-1 knock-out
Cfu - colony forming unit
CLP - cecal ligation and puncture
CR - complement receptor 3
D5W - 5% dextrose in water
DAP - diaminopropane
DMSO - dimethyl sulfoxide
G-CSF - granulocyte colony stimulating factor
GI - gastrointestinal tract
GM-CSF - granulocyte monocyte colony stimulating factor
GP - glucan phosphate
h - hour
H and E - hemotoxylin and eosin
IFNγ - interferon gamma
IL - interleukin
IP - intraperitoneal
ITAM - tyrosine based activation motif
IV - intravenous
LPS - lipopolysaccharide
m - minute
MβCD - methyl beta cyclodextran
µg - microgram
mg - milligram
µl - microliter
ml - milliliter
mM - millimolar
NF-κB - nuclear factor kappa B
PAMP - pathogen associated molecular pattern
PAS - periodic acid Schiff
PBS - phosphate buffered saline
PEG - polyethylene glycol
PI3K - phosphoinositide 3 kinase
PRR - pattern recognition receptor
SIRS - systemic inflammatory response syndrome
SR-A - scavenger receptor A
SYK - spleen tyrosine kinase
TLR - toll like receptor
TNFα - tumor necrosis factor alpha
TRITC - rhodamine
WT - wild type
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Oral delivery and gastrointestinal absorption of soluble glucans stimulate increased resistance to infectious challenge.
*Journal of Pharmacology and Experimental Therapeutics*, 314(3), 1079-1086.

Modulation of the phosphoinositide 3-kinase pathway alters innate resistance to polymicrobial sepsis.
*Journal of Immunology*, 172, 449-456.

Honors and Awards:
Second Place in the 21st Annual Student Research Forum, East Tennessee State University, Johnson City, Tennessee, 2005
Inducted as a member of Phi Zeta Veterinary Honors Society, University of Tennessee College of Veterinary Medicine, Knoxville, Tennessee, 1997
Cum laude graduate of Tennessee Technological University, Cookeville, Tennessee, 1994