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**EFFECTS OF THE MEDICINAL HERB, *PANAX NOTOGINSENG*, ON THE
FATE AND FUNCTION OF PROFESSIONAL ANTIGEN PRESENTING CELLS**

By

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Dissertation

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for the degree of

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EFFECTS OF THE MEDICINAL HERB, *PANAX NOTOGINSENG*, ON THE FATE AND FUNCTION OF PROFESSIONAL ANTIGEN PRESENTING CELLS

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Abstract

Antigen presenting cells (APCs) perform the essential task of integrating responses between the innate and adaptive immune system. Several approaches have been undertaken to manipulate the effects of APCs for therapeutic purposes. *Panax notoginseng* is a medicinal herb that is purported to possess a number of properties including modulation of the immune system. However, limited information exists on the effects and toxicities of this herbal on APCs. In this regard, we assessed the effects of *Panax notoginseng* on the fate and function of professional APCs in murine models using macrophages and dendritic cells (DCs). APCs were stimulated with the toll-like receptor ligands LPS, CpG and poly(I:C) and treated with notoginseng (0-200 µg/ml). The LPS induced levels of the proinflammatory cytokine TNF- α , as well as the expression of accessory molecules MHC II, CD40 and CD86, were decreased dependent on notoginseng exposure time-points relative to LPS stimulation. LPS induced IL-1 β , IL-6 and IL-12 production was also decreased with concurrent notoginseng treatment for 24 hours. Notoginseng decreased TNF- α and CD40 activation by CpG and poly(I:C), but had varied effects on the induction of IL-6 and CD86. Furthermore, treatment of APCs with ginsenosides Rb1 and Rg1 had differential effects on the production of TNF- α and IL-6. Phagocytosis of FITC-conjugated ovalbumin antigen by DCs was decreased by notoginseng. Furthermore, the uptake of FITC-conjugated modified LDL was reduced in notoginseng treated DCs. However, T cell proliferation in response to notoginseng-treated-antigen-loaded DCs was not affected *in vitro* or *in vivo*. Mechanistically, notoginseng reduced nuclear levels of the transcription factor NF κ B, but had no effect on glucocorticoid receptor activation. No immunotoxicities were observed with low dose notoginseng (660 µg/kg) treatment of Balb/c mice *in vivo*. Collectively, our results indicate that notoginseng decreased inflammatory mediator production by APCs, without altering their ability to induce antigen specific CD 4⁺ T cell proliferation. Our research provides insight into the potential use of this herbal in the treatment of inflammatory diseases as a safe and effective complement to existing remedies.

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CHAPTER 1

Introduction

This study describes the effects of the medicinal herb *Panax notoginseng* (notoginseng) on the fate and function of professional antigen presenting cells (APCs). This introductory chapter will (1) outline the organization and function of the immune system with primary focus on APCs, Toll-like receptors (TLRs) and inflammation, (2) provide detailed information about the general properties and uses of plants in the Ginseng species, and (3) elucidate modes by which notoginseng modulates immune function.

Overview of the Immune System

The immune system is designed to eliminate any object that is characterized as being foreign or a danger to the body (Goldsby et al., 2003). This includes protection from invading microorganisms such as bacteria, viruses and fungi, as well as eliminating cells with altered proteins or other molecules that are no longer recognized as “self”. Surveillance of an individual involves a complex process, with various hierarchies and checkpoints existing to facilitate immune efficiency. These hierarchies begin with the immune system being divided into two sections, innate and adaptive immunity (Parkin and Cohen, 2001). These sections are not mutually exclusive, but work in conjunction with each other to effectively eliminate threats to the body. The innate arm of immunity recognizes non-specific threats and reacts immediately to danger patterns and signals. Alternatively, the adaptive arm is the precision arm and recognizes specific molecules that are capable of binding to adaptive immune cell receptors. These molecules are

known as antigens. However, as specificity takes time, there is a lag between the immediate, but non-specific, innate response and the precise, but slower, adaptive immune response.

Innate immunity

Innate immunity is composed of four types of defensive barriers: anatomic, physiologic, phagocytic and inflammatory (Goldsby et al., 2003). The anatomic barrier encompasses the skin and mucous membranes. This barrier is the first line of defense and prevents the entry of microorganisms into our body. Physiological barriers include the body's ability to control temperature, pH and chemical mediators whose primary function is to target pathogens and create an environment that is not conducive to their growth. The third line of innate defense mechanism is the ingestion (phagocytosis) and destruction of pathogens by phagocytes. Specialized immune cells such as monocytes, macrophages, neutrophils and dendritic cells conduct phagocytosis. There are also immune cells whose primary function is to present pieces of phagocytosed pathogens, known as antigens, to adaptive immune cells. These cells are known as antigen presenting cells (APCs) and will be discussed further in detail later in this chapter. The fourth defensive strategy used by our innate cells is the production of an inflammatory environment. This environment is created when leukocytes and inflammatory molecules converge on sites of infection or tissue injury. While this process is advantageous to clearing insults to tissue injuries, persistence of an inflammatory response can lead to chronic inflammatory diseases. The implications of inflammation and an overactive immune response will also be discussed below.

Antigen presenting cells (APCs)

APCs are specialized immune cells that express major histocompatibility complex (MHC) II molecules on their cell surface and can process and present foreign substances known as antigens, which binds to these molecules to elicit adaptive immunity (Goldsby et al., 2003). These MHC II molecules are important because T cells recognize antigens only when bound to a MHC complex. This presentation of antigens by APCs to T cells is widely referred to in immunology as “signal 1”. The “second signal” involves APCs delivering co-stimulatory signals to T cells that are essential for their full activation. These two processes are known in immunology as the two-signal hypothesis (Fig. 1-1). In addition to these signals, activated APCs also release a third signal known as cytokines. Cytokines are small protein molecules that mediate numerous physiological responses including the development of specific T cell responses, modulation of inflammation, regulation of hematopoiesis and control of cell proliferation and differentiation (Goldsby et al., 2003). The activation process begins when an APC encounters antigen in the periphery. Uptake of antigens can trigger the migration of certain APC types to a secondary lymphoid organ. There, antigen-bearing APCs select antigen-specific lymphocytes from a pool of recirculating T cells. Under the right conditions, interaction of this APC with T cells initiates an adaptive immune response.

The three major types of APCs in order of importance are dendritic cells (DCs), macrophages and B cells. DCs are known as the primary professional APCs of the immune system (Trombetta and Mellman, 2005). These cells are unique for a number of reasons the main one being that DCs are the most potent APCs capable of inducing the T cell-mediated immunity, thus permitting the establishment of immunological memory

(Banchereau et al., 2000c; Foged et al., 2002; Gilboa and Vieweg, 2004). DCs constitutively express high levels of MHC molecules, as well as high levels of the costimulatory molecules CD80 and CD86 that are needed to initiate the T cell response. As a result, DCs are potent activators of naïve, memory and effector T cells. Under different microenvironments DCs are able to induce contrasting states of immunity or tolerance.

DCs mainly reside in peripheral tissues. Upon encountering a danger signal, these cells mature into potent APCs and migrate to draining lymph node organs where they can interact with T cells (Kooten van and Woltman, 2004). The maturation process results in decreased capacity to capture antigen, but increased expression of the activation molecules CD40, CD86 and MHC II (Kooten van and Woltman, 2004). These changes in DC morphology are important for increasing their interactions with corresponding receptors on the surface of T cells to facilitate activation of the adaptive immune system.

The major types of DC subsets are lymphoid and myeloid, in addition, interstitial and Langerhans DC subsets maybe also be present dependent on animal type (Banchereau et al., 2000c). In previous years, there have been numerous studies on a new type of DC being present in both species, now known as plasmacytoid DCs (Colonna et al., 2004; Zhang and Wang, 2005). All DC subsets differ in phenotype, localization and function. The traditional DC subsets express appreciable levels of the classic cell surface molecules MHCII and CD11c by which they can be identified by using tagged antibodies against these proteins. However, plasmacytoid DCs do not express CD11c in humans and express it at very low levels in mice (Colonna et al., 2004).

DCs can be differentiated and expanded from progenitor cells by using the growth factors FMS-like tyrosine kinase 3 ligand (Flt3-L) and granulocyte macrophage-colony stimulating factor (GM-CSF) (Banchereau et al., 2000c). Flt3-L targets primitive hematopoietic bone marrow progenitors, inducing their expansion and differentiation with all DC subsets increasing dramatically. In contrast, GM-CSF preferentially expands myeloid DC subsets. Unlike DCs, macrophages do not express MHC II and costimulatory molecules constitutively, but induces them after their activation by phagocytosis of microbes such as bacteria or viruses. This means that while DCs are the most important cells in naïve T cell and B cell activation, unstimulated macrophages do not activate these cells as efficiently. In addition to antigen presentation, macrophages influence a range of immune responses by antigen recognition, capture, clearance and transport (Gordon, 1998). The function and activity of macrophages is strongly influenced by their microenvironment (Gordon, 1998; Laskin et al., 2001). The liver and lung have the largest populations of macrophages in the body (Laskin et al., 2001). As a result of the unique attributes of these tissues, hepatic and pulmonary macrophages play essential roles not only in nonspecific host defense mechanisms, but also in the homeostatic responses of these tissues (Laskin et al., 2001).

The third type of professional APCs is the B cell. Among the APCs B cells are least likely to uptake and present antigens. In fact, they are better known for their adaptive immune functions as opposed to their APC capability. As such, their functions will be discussed in the section on adaptive immunity later in this chapter.

Figure 1-1.

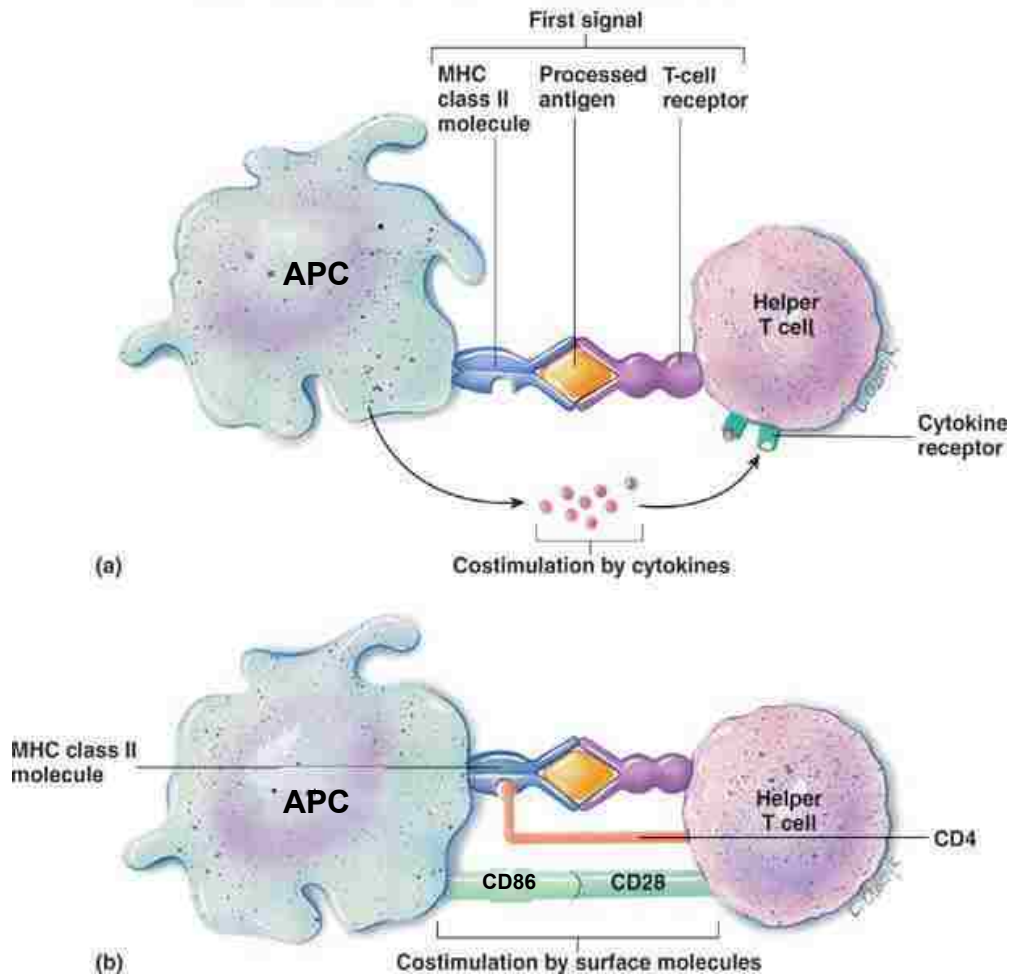


Figure 1-1. The two signal hypothesis of T cell activation. APCs activate helper T cells by the binding of the MHC II/antigen complex to the T cell receptor. This process is known as signal 1. Signal 2 involves the binding of costimulatory molecules on the APC such as CD86 with CD28 on the helper T cell. Other signals such as cytokine production by the APC also aid in the activation process. This figure was adapted from Seely et al. 2003, *Anatomy and Physiology*, Mc-Graw Hill Companies, pg 805.

Toll-like receptors (TLRs)

Certain types of molecules are unique to microbes and are never found in multicellular organisms. Innate immune cells have evolved to recognize these pathogen associated molecular patterns (PAMPS) using pattern recognition receptors (PRRs) (Doyle and O'Neill, 2006). PAMPS are highly conserved within microbial species, but are generally absent from human cells. Of these classes of receptors, the TLRs are among the most important. The TLR family of proteins is an ancient one, recognizing generations of defensive responses to pathogens in organisms as widely separated in evolution as humans and flies. Over ten different types of TLRs have been recognized, each being activated by a wide array of PAMPs (Fig.1-2). These TLRs are characterized by the presence of an extracellular leucine-rich repeat domain (LRR) and an intracellular Toll/IL-1 receptor (TIR) domain (Carpenter and O'Neill L, 2007). In mammals, many TLR receptors are found on the cell membranes of innate immune cells such as macrophages and DCs. However, TLR3, 7, 8 and 9 are expressed in intracellular compartments, principally in the endosomes and the endoplasmic reticulum (Carpenter and O'Neill L, 2007; Kanzler et al., 2007). TLRs are either constitutively expressed or induced in different cell types, which determines their capacity for microbial detection (Napolitani et al., 2005). TLRs are differentially coupled to a number of cell signaling pathways. However, with the exception of TLR3, all TLRs are coupled to the myeloid differentiation primary-response protein 88 (MyD88) adaptor; TLR3 and TLR4 are also coupled to TIR-related adaptor protein inducing interferon (TRIF) (Kanzler et al., 2007; Napolitani et al., 2005). Signaling through TLRs leads to the activation of transcription

Figure 1-2.

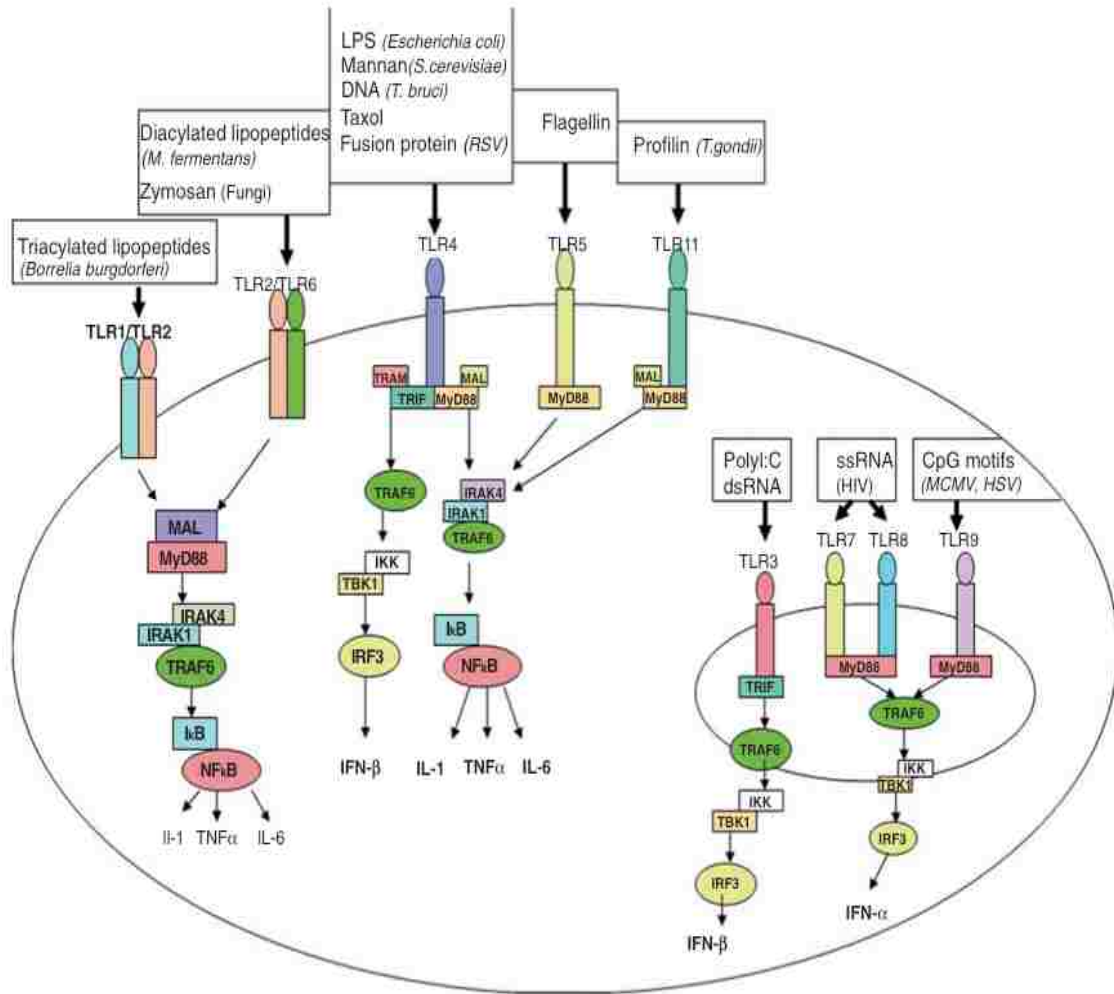


Figure 1-2. The initiation of the immune responses by TLRs activated by microbial ligands. TLR signaling is initiated by distinct PAMPS. This activation ultimately leads to inducing downstream transcription factors responsible for initiating a variety of immune mediators. This figure was obtained from (Carpenter and O'Neill L, 2007).

molecules including nuclear factor kappa B (NFκB) and interferon response factor 3 (IRF-3) which in turn culminates in activation and synthesis of a range of pro-inflammatory mediators including cytokines, co-stimulatory molecules and inflammatory enzymes (Doyle and O'Neill, 2006). Recent studies on TLRs also include enhanced roles that these receptors play in immune cell physiology such as enhanced antigen capture via induction of actin remodeling of the cytoskeleton of DCs (West et al., 2004). As a result of these unique immunomodulatory properties of TLRs, new trends in medicine have sparked interest in the development of TLR agonists in the treatment of cancer, viral infections and as an adjuvant in potent new vaccines (Kanzler et al., 2007; Wickelgren, 2006). In addition, as recognition of inappropriate TLR responses in autoimmunity and inflammation grows, efforts have begun to develop antagonists to Toll-like receptors as a treatment for immune related diseases (Kanzler et al., 2007; Marshak-Rothstein and Rifkin, 2007).

Inflammation

Tissue damage from an injury or by an invading microorganism induces a complex sequence of events collectively known as the inflammatory response. The response is simply characterized as involving redness, swelling, heat and pain, which are classic symptoms from the three major events in inflammation. These events are vasodilation, increased capillary permeability and influx of phagocytes from the capillaries into the damaged tissue to attack pathogens. Inflammation allows immune cells to quickly and efficiently travel to sites of infection, where they can target and eradicate pathogens.

While the immune system is important for protecting us against pathogens and opportunistic organisms, there are some incidences where it is not kept in check. This condition is known as autoimmunity and primarily involves a hyperactive immune response to the body's own antigens. Autoimmunity is primarily characterized by chronic symptoms of inflammation which causes discomfort and leads to many different disease conditions such as rheumatoid arthritis, diabetes mellitus type 1 and lupus erythematosus. In addition to autoimmune diseases there are other conditions such as atherosclerosis, with components of inflammation being primarily responsible for exacerbation of the diseases (Vanderlaan and Reardon, 2005). In atherosclerosis, both the innate and adaptive immune system participate in the exacerbation of the disease. Macrophages and vascular smooth muscle cells are involved in retaining and modifying lipids in the vessel walls, as well as driving the chronic vascular inflammation that characterizes this disease (Vanderlaan and Reardon, 2005).

There are many natural and synthetic agents that are currently used in the treatment of inflammatory diseases. In spite of this, there is still a constant search for better agents to treat inflammation. Presently, anti-inflammatory agents target many aspects of the immune system including cytokines, inflammatory enzymes and transcription factors responsible for inducing or decreasing the production of these inflammatory mediators. The most widely used are the non-steroidal anti-inflammatory agents (NSAIDs) and steroidal anti-inflammatory agents, primarily the glucocorticoid receptor (GCR) agonists. These agents can also target activation of the transcription factor, NF κ B.

Nuclear Factor Kappa B (NFκB)

NFκB is a family of transcription factors involved in stress-induced, immune and inflammatory responses (Dixit and Mak, 2002). These molecules also play important roles in the development of hematopoietic cells and lymphoid organs (Dixit and Mak, 2002). NFκB is described as being one of the most studied transcription factors in biology (Dixit and Mak, 2002).

Members of the NFκB family are formed by a dimeric combination of subunits and are activated by a number of receptor-mediated signaling pathways (Dixit and Mak, 2002). There are five members of this family of transcription factors, Rel (c-Rel), Rel A (p65), Rel B, NFκB1 (p105/p50) and NFκB2 (p100/p52). These proteins work together to regulate the expression of genes that encode cytokines, chemokines and adhesion molecules thereby coordinating adaptive and immune responses. NFκB/Rel dimers interact with a group of inhibitory proteins called the IκB family. These proteins anchor NFκB complexes in the cytoplasm in its inactive form (Karin and Ben-Neriah, 2000b). The degradation of an IκB complex initiated by an inflammatory stimulus leads to the translocation of NFκB into the nucleus. Inside the nucleus, NFκB complex can bind to DNA to induce the transcription of immune and inflammatory genes.

Recently, there have been a number of studies on agents that target the activation of NFκB (Tanaka et al., 2007). From a biomedical standpoint, controlling the activity of this transcription factor is a prime target for modulating a wide array of immune and inflammatory genes in numerous diseases (Celec, 2004b).

Adaptive immunity

As mentioned before, innate immune cells also deliver messages to adaptive immune cells so they can aid in the fight against pathogens. These adaptive immune cells are capable of selective recognition and elimination of pathogens. Lymphocytes exclusively display characteristics of antigen specificity, diversity, immunologic memory and self/nonself recognition. They distinguish subtle differences among antigens, recognize a tremendous number of unique structures, remember a pathogen for a faster response time upon a secondary encounter and can tell the difference between self and foreign molecules. Adaptive immunity is mediated by two types of cells, T lymphocytes and B lymphocytes (Fig 1-3).

B cells

B cells are the antibody producing cells of the immune system (Parkin and Cohen, 2001). Antibodies are glycoprotein molecules with tremendous specificity for antigens. These glycoprotein molecules are the mediators of humoral immunity. Mature B cells leave the bone marrow expressing membrane-bound immunoglobulin antibody (Fagarasan and Honjo, 2000). These naïve B cells circulate in the blood and lymph and are eventually carried to the spleen and lymph nodes where they reside. When an antigen specific to its membrane-bound antibody activates a B cell, it can proliferate and differentiate into antibody secreting cells known as plasma cells or form long-lived memory B cells. During this stage, affinity maturation and class switching occurs. Affinity maturation increases the average affinity of the antibody for a specific antigen while class switching involves a change in the "effector" isotype of the antibody that the B cell produces.

Figure 1-3.

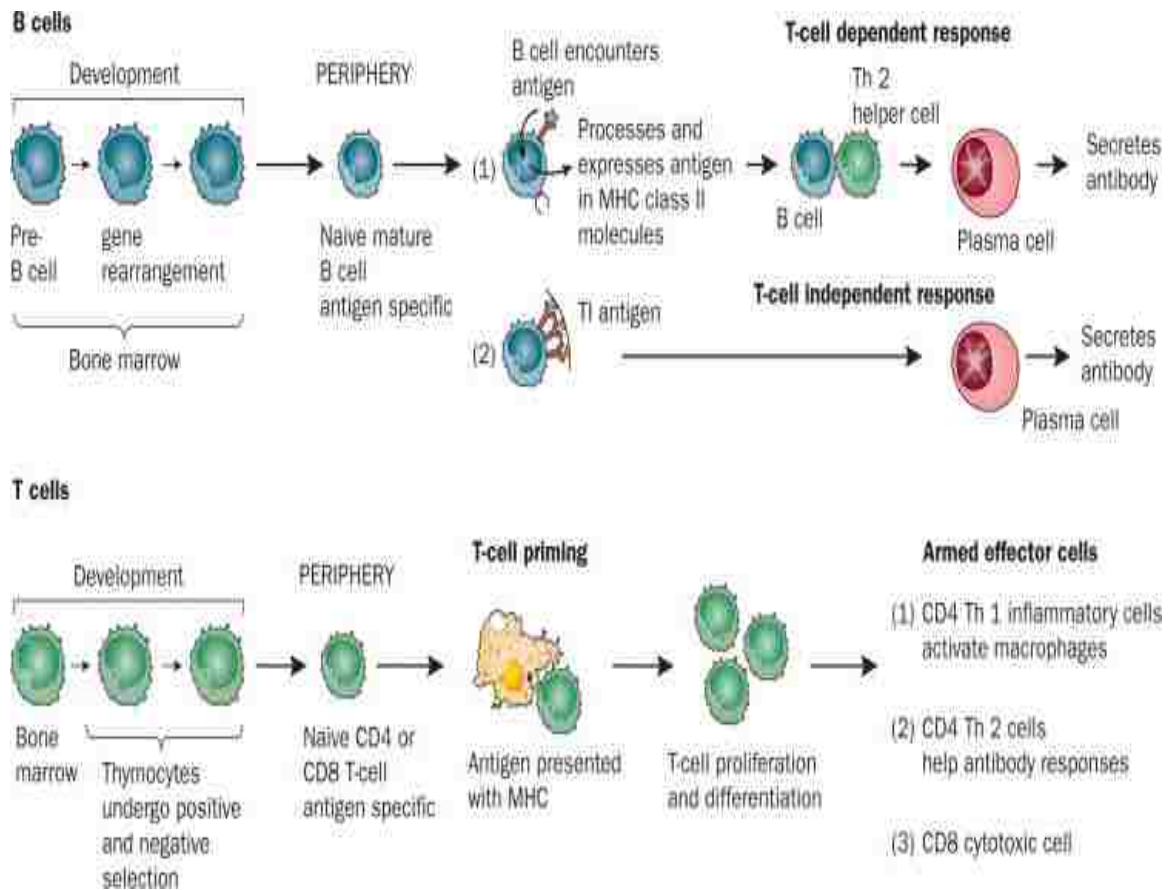


Figure 1-3. The role of T and B lymphocytes in immunity. An outline of B and T cells from development to activation, and their effector responses. This figure was obtained from (Parkin and Cohen, 2001).

Depending on the nature of the antigen, B cell activation can occur via two different routes, namely T cell-dependent and T cell-independent responses. The T cell-dependent response requires direct contact with T helper (T_h) cells. This process entails antigens binding directly to the B cell surface immunoglobulin (Ig), along with costimulation by antigen-specific T cells through CD40-CD40 ligand interaction and the secretion of cytokines (Fagarasan and Honjo, 2000). This is followed by appropriately activated B cells proliferation and differentiation into plasma cells and memory cells. T cell-dependent immune responses usually involve conventional (B2) B cells. However, another subset of B cells, B1 cells, along with marginal zone B cells are primarily responsible for producing antibodies in a T-independent manner (Fagarasan and Honjo, 2000). These cells recognize common bacterial antigens such as LPS as well as self-antigens, such as phosphatidylcholine, DNA and membrane proteins on erythrocytes and thymocytes. In T cell-independent response by B1 cells, these antigens can activate B cells in the absence of direct participation with T cells. Instead, B cell receptors are effectively cross-linked by antigens with repetitive epitopes, to produce large amounts of antibodies. These antibodies are usually of lower affinity than those produced by T cell-dependent responses.

In addition to their primary function as the B cell receptors, antibodies contribute to targeting proteins on pathogens for elimination by a number of methods. This includes neutralizing pathogens and opsonization of antigens to enhance clearance of microbes from the body. The primary properties of antibodies have also led to their use in a variety of biological industries including research labs and even in the synthesis of many pharmaceutical agents.

As previously mentioned, B cells are also considered as professional APCs. However, of the three professional APCs, B cells are the ones least likely to uptake and present antigens. This results from a number of factors including the low frequency of naïve B cells that bear a receptor for a particular antigen, their absence from skin and mucosal sites through which most pathogens and environmental antigens enter the body, and their dependence on help from activated CD4 T cells (Rodriguez-Pinto, 2005). In addition to these factors, even though resting B cells express MHC II molecules, the co-stimulatory molecule CD86 still has to be induced before they can adequately activate T cells. However, upon antigen specific activation, B cells become very good APCs, expressing the necessary accessory/costimulatory molecules and can stimulate naïve, memory as well as effector T cells.

T cells

Unlike B cells, T cells can only recognize antigen that is bound to MHC molecules on an APCs. MHC molecules are not important to T cells in antigen recognition only, but also play a major role in T cell development in the thymus. Antigenic diversity of the T cell population is limited during maturation by a selection process that only allows MHC-restricted and non-self reactive T cells to mature. The final maturation stages in the development of most T cells proceed along two different pathways, generating functionally distinct subpopulations of CD4⁺ and CD8⁺ T cells. These populations recognize different MHC complexes, with CD4⁺ cells and CD8⁺ cells being MHC II- and MHC I- restricted, respectively.

Activation of mature T cells begins with the recognition of a specific antigen in the context of MHC molecule on an APC, a process known as signal 1 as described in the

previous section on APCs. In order to be fully activated, there must also be a second signal involving the interaction of various accessory molecules on the T cell with their corresponding molecules on the APCs. Following these two signals are a number of pathway activations leading to the transcription of effector molecules including the cytokine IL-2, which was originally described as the T cell growth factor (Vincenti and Luggen, 2007). IL-2 production is sometimes referred to as signal 3 as it induces the proliferation of T cells into various effector as well as memory T cells when combined with antigen/MHC molecules and costimulation (Vincenti and Luggen, 2007).

While the activation and differentiation of both CD4⁺ (T helper-T_h) and CD8⁺ (T cytotoxic-T_c) cells are vastly similar, there are dramatic differences in the effector functions of these subsets. T_h cells are usually the first T cells activated in an immune response. These cells orchestrate adaptive immune responses by either primarily activating B cells for a humoral response or T_c for a cell mediated response. Differences in the pattern of cytokines secreted by activated T_h results in these different types of immune responses.

As such, T_h cells can be classified into two subpopulations distinguished by these different panels of cytokines which they secrete (Dong and Flavell, 2001). These populations are referred to as the T_h 1 and T_h 2 subsets. The T_h 1 subset secretes the cytokines IL-2, IFN- γ , TNF- α and mediates cellular immunity. These events are initiated by the transcription factor T-Bet which is primarily induced by the presence of high levels of IL-12 and IL-18 produced by APCs (Gutcher and Becher, 2007). In contrast, T_h 2 subsets secrete IL-4, IL-5, IL-10 and IL-13 and regulate humoral immune responses. T_h 2 subsets are promoted by GATA-3 transcription factor activation by IL-4 secretion by

naïve CD4⁺ T cells. Of additional importance is that cytokines, secreted by a particular T_h subset, are able to further promote the expansion of that subset population while inhibiting the development of the other T_h population (Gutcher and Becher, 2007).

Effector T cell responses need to be controlled after completing its mission of eradicating pathogens, in order to prevent it from inducing autoimmune conditions (Beissert et al., 2006). The T regulatory (T regs) cell subtype, are the primary cells responsible for managing this process. These cells were first described in the early 1970s as suppressor T cells (Beissert et al., 2006). However, their existence was questioned and the presence of these cells in the immune system was dismissed (Wickelgren, 2004). Their presence was revived in the 1990s with an abundance of research in the field leaving no doubt as to their existence. Since then, there has been continued research on defining lineage specific markers for these cells, with the front-runners being CD4⁺/CD25⁺ and Fox P3⁺ T cells. T regs can suppress T cell responses by tolerizing T cells in certain conditions. It is believed that the control of T cell responses by T regs will provide clues in the management of numerous immune disorders (Wickelgren, 2004).

Ginseng

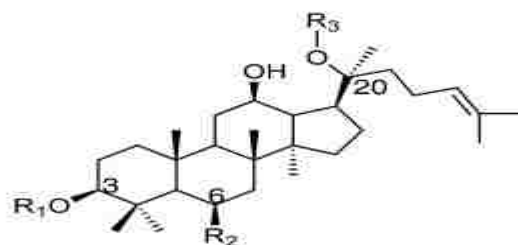
In 1994 the United States Congress passed the Dietary Supplement Health and Education Act to promote consumer access to dietary supplements (Engel and Straus, 2002). This law defined a new category of food — “dietary supplement” — which would include herbs and other botanicals, vitamins and minerals, and other substances (Engel and Straus, 2002). Since the passage of this act, the dietary supplement industry has experienced considerable growth (Noonan and Patrick Noonan, 2006). However, the

marketing of these products as food also opened the door for their sales without standardized test to prove whether they are safe, efficacious or produced under current good manufacturing processes (CGMP). As a result, on June 25, 2007, the Food and Drug Administration (FDA) issued guidelines which ensures that dietary supplements are produced in a quality manner, do not contain contaminants or impurities and are accurately labeled. While these rules will enable the application of CGMP that provides some measure of protection for the public, it does not adequately address whether these products are biologically safe or effective.

Currently, ginseng is one of the most popular dietary supplements, with an annual sale of over USD 200 million (Yue et al., 2007). Ginseng is a deciduous perennial plant that belongs to the Araliaceae family. Presently, twelve species of Ginseng have been identified. Among them, *Panax ginseng* C. A. Meyer, *P. quinquefolium* L and *P. notoginseng* are the three most well known and scientifically investigated species. Ginseng is purported to have numerous pharmacological and therapeutic properties. It affects the central nervous system (CNS), cardiovascular system, endocrine secretion, immune function, metabolism, and has anti-stress and anti-aging actions. These properties have been primarily attributed to the ginsenoside components of Ginseng. However, studies also suggest that phytosterols, peptides, polysaccharides, fatty acids, polyacetylenes, vitamins and mineral components also contribute to Ginseng's, biological effects (Buettner et al., 2006).

Ginseng has been used clinically in China, Korea and Japan for thousands of years (Radad et al., 2006; Yue et al., 2007). In the 18th century, the West recognized the medicinal advantages of Ginseng. This subsequently led to a large number of

Figure 1-4.



Ginsenoside	R ₁	R ₂	R ₃
<i>Protopanaxatriol</i>			
Rg₁	H	-O-glc	glc
Rg₂	H	-O-glc(2-1)rha	H
Rc	H	-O-glc(2-1)rha	glc
Rf	H	-O-glc(2-1)glc	H
Rh₁	H	-O-glc	H
F1	H	-OH	glc
<i>Protopanaxadiol</i>			
Rb₁	glc(2-1)glc	H	glc(6-1)glc
Rb₂	glc(2-1)glc	H	glc(6-1)ara(p)
Rg₃	glc(2-1)glc	H	H
Rh₂	glc	H	H
Rc	glc(2-1)glc	H	glc(6-1)ara(f)
Rd	glc(2-1)glc	H	glc
Ra	glc(2-1)glc	H	glc(6-1)glc
F2	glc	H	glc
Compound Y	H	H	glc(6-1)ara(p)
Compound K	H	H	glc
Compound O	glc	H	glc(6-1)ara(p)
Compound Mc	glc	H	glc(6-1)ara(f)

Figure 1-4. The chemical structure of ginsenosides. *glc* = glucosyl (C₆H₁₁O₆⁻); *rha* = rhamnosyl (C₆H₁₁O₅⁻); *ara* = arabinosyl (C₅H₉O₅⁻); p = pyran; f = furan. This figure was obtained from (Yue et al., 2007).

investigations being conducted on its botany, chemistry, pharmacology and therapeutic applications (Yue et al., 2007).

Ginsenosides

As previously mentioned, the most prominent and pharmacologically active constituents of ginseng are the saponin glycosides known as ginsenosides (Yue et al., 2007). Over thirty ginsenosides have been isolated and characterized from various Ginseng species (Fig. 1-4) (Buettner et al., 2006). In general, the concentration and types of ginsenosides present in a Ginseng plant vary widely, depending on the species, age and part of the plant used, and even the preservation or extraction method (Yue et al., 2007). It is therefore possible that products containing the same amount of total ginsenosides could contain different individual ginsenoside compositions and ratios (Buettner et al., 2006).

The variability in ginsenoside content in Ginseng contributes to the heterogeneity of reported findings in the research of this herbal (Buettner et al., 2006). This is because different ginsenosides have different pharmacological effects which can sometimes enhance or antagonize observed medicinal effects depending on their concentrations in a particular species (Buettner et al., 2006). Ginsenosides are usually classified into three major categories, namely protopanaxadiols (PPD) (e.g. Rb₁, Rb₂, Rc, Rd, Rg₃, Rh₂), protopanaxatriols (PPT) (e.g. Re, Rf, Rg₁, Rg₂, Rh₁) and the oleanolic acid derivatives (Yue et al., 2007). These compounds have a steroid-like skeleton consisting of four *trans*-rings, with differences from each other being dependent on the type (e.g. glucose, maltose and fructose), number of sugar moieties and the sites of attachment of the hydroxyl group (Yue et al., 2007). Ginsenosides are amphipathic in nature with the

hydroxyl (-OH) groups allowing both interactions between the polar head of phospholipid membranes and the β -OH group of cholesterol, and the hydrophobic steroid backbone interacting with the hydrophobic side chains of fatty acids and cholesterol.

Ginsenosides are thought to exhibit their actions through a number of mechanisms including signaling through the plasma membrane, cytosol, or even in the nucleus. They can initiate binding to membrane receptors such as ATPase pumps, ion transporters and channels, voltage gated channels and G-proteins and subsequently activate associated downstream signaling cascades (Yue et al., 2007). As they are amphipathic, they can intercalate into plasma membranes resulting in alterations in membrane fluidity, thereby triggering a series of cellular responses. Ginsenosides can also potentially bind to a number of intracellular steroid hormone receptors including glucocorticoid receptor (GCR), estrogen receptor (ER), progesterone receptor (PR), androgen receptor (AR) and mineral corticoid receptor (MR), using their hydrophobic 'steroid-like' backbone (Fig.1-5) (Yue et al., 2007). This can lead to the regulation of gene transcription by binding with the specific gene response elements.

Absorption, Distribution and Metabolism

After oral ingestion, ginsenosides pass through the stomach and small intestine and into the large intestine without decomposition by either gastric juice or liver enzymes (Fig. 1-6) (Hasegawa, 2004) . However, upon arrival into the large intestines, ginsenosides may be deglycosylated by colonic bacteria prior to absorption and transit to the circulation. Studies in rats suggest that the oral bioavailability of the intact ginsenoside is extremely low, varying from 0.1-18 % depending on the type of ginsenoside tested

Figure 1-5.

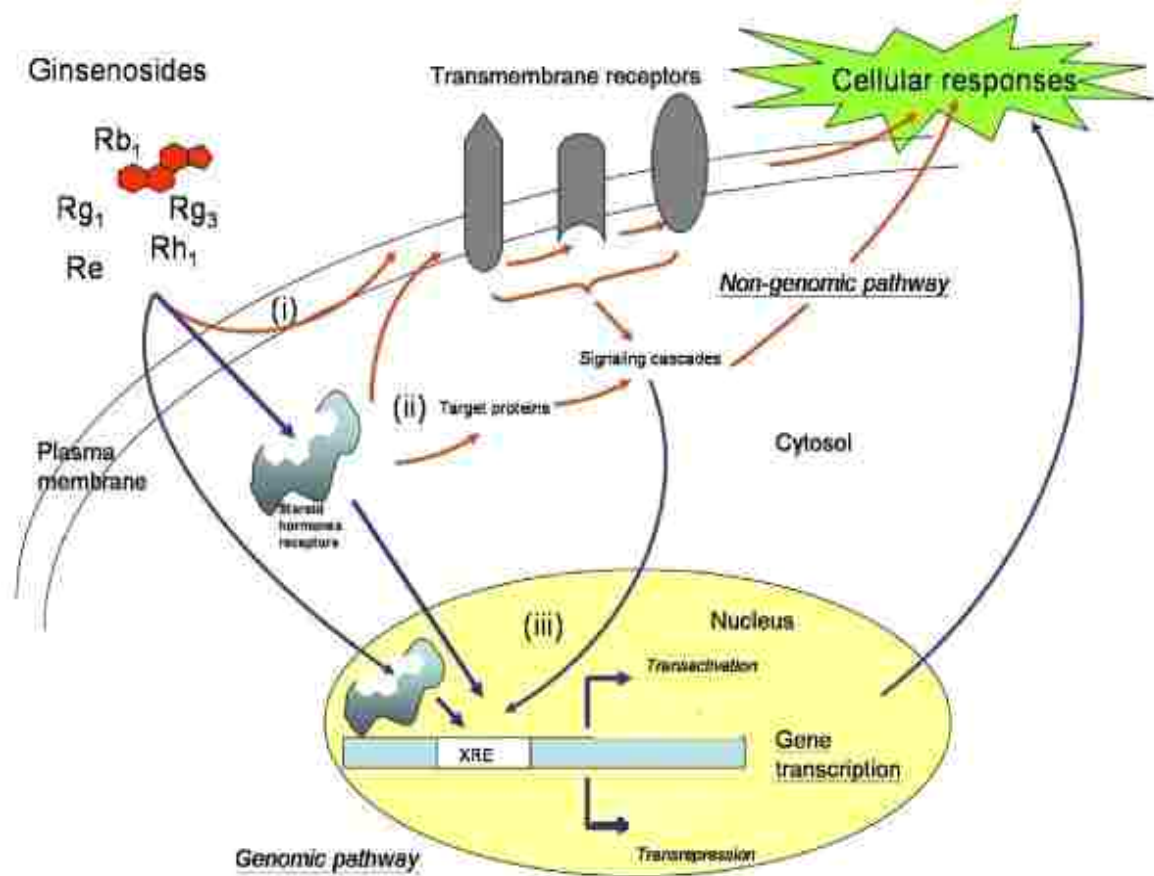


Figure 1-5. Schematic overview of ginsenoside-mediated genomic and non-genomic pathways. Ginsenosides possess a steroid-like skeleton composed of four *trans*-rings with different degrees of glyco-substitution. As ginsenosides are amphipathic in nature they can exhibit their actions at different cellular locations, such as the plasma membrane, cytosol and nucleus. Through the non-genomic pathway, (i) they can initiate their actions by binding with the transmembrane receptors, intercalating into the plasma membrane resulting in an alteration of membrane fluidity and (ii) binding with steroid hormone receptors (SHRs) present inside or outside the nucleus. (iii) the ligand-bound SHRs can translocate into the nucleus, where they regulate gene transcription by binding with the specific Response Elements (XRE). This is the so-called 'genomic pathway'. Consequently, the altered gene products can affect the final cellular responses. This figure was adapted from (Yue et al., 2007).

Figure 1-6.

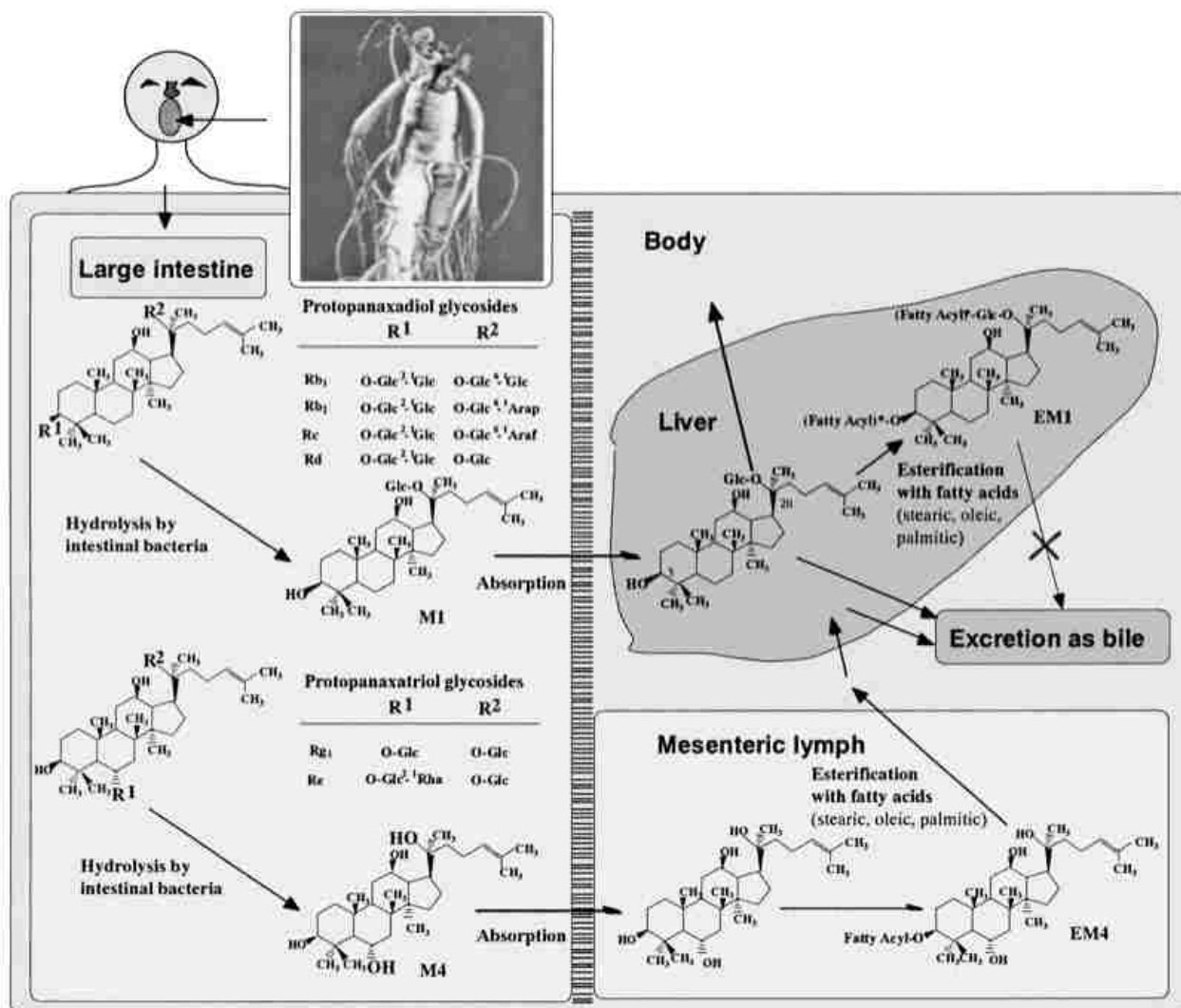


Figure 1-6. Metabolic Activation of Ginsenosides.

Putative metabolic pathways of ginsenosides in the body after oral administration. Ginsenosides are deglycosylated to M1 or M4 by intestinal bacteria. This is followed by absorption into the blood or mesenteric lymphatics. Although most of M1 is excreted as bile, some M1 may be esterified with fatty acids at C-3 of the aglycone moiety or C-6 of the glucose moiety in the liver. EM1 is not excreted in the small intestine and accumulates in the liver longer than M1. However, most M4 is esterified with fatty acids and accumulates in tissues including the liver and lung. This figure was obtained from (Hasegawa, 2004).

(Hasegawa, 2004). In the large intestines, colonic bacteria cleave the oligosaccharide connected to the C-3 or C-20 hydroxyl group of the aglycone stepwise from the terminal sugar (Hasegawa, 2004).

Thirteen different metabolites might be formed from this process with 20S-protopanaxadiol 20-O- β -D-glucopyranoside (M1) and 20S-protopanaxatriol (M4) being the major two (Hasegawa, 2004). Interstitial bacteria responsible for ginsenoside metabolism include, *Prevotella oris*, *Eubacterium A-44*, *Bifidobacterium K506*, *Bacteroides JY6*, and *Fusobacterium K-60*.

Following absorption from the intestines, metabolites can be further esterified with fatty acids by the liver. The resultant fatty acid conjugates are still active molecules that are sustained longer in the body than parental metabolites (Hasegawa, 2004). It is of importance to note that intestinal bacteria are changeable dependent on host conditions including diet, health and even stress. Bacterial ginsenoside-hydrolyzing potentials can differ among humans and experimental mice (Hasegawa, 2004). Therefore, it is possible that the individual differences in bacterial ginsenoside-hydrolyzing potentials account for differences in Ginseng efficacy. Additionally, although the absorption, distribution and metabolism of ginsenosides has been studied in animals and *in vitro*, knowledge concerning the systemic availability of ginsenosides and their degradation products in humans is still vastly limited and is mostly inconclusive (Tawab et al., 2003).

The effects of Ginseng on the immune system

One of the primary uses of Ginseng is to modulate immune function (Kiefer and Pantuso, 2003). Ginsenosides as well as the polysaccharide components of Ginseng have been shown to reduce inflammation in various experimental models (Ahn et al., 2006).

Numerous experiments have demonstrated decreased inflammatory mediator production by activated innate immune cells after Ginseng treatment. This includes decreased cytokine production by macrophages and mast cells (Hofseth and Wargovich, 2007; Kim et al., 2007). In addition, decreased expression and production of inducible inflammatory enzymes such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in innate immune cells were demonstrated in a number of studies (Kim et al., 2007).

Limited information exists describing the effects of Ginseng or its derivatives on DCs. In the one study published on human DCs, there was an increase in maturation signals after treatment with M1 and M4 metabolites of Ginseng (Takei et al., 2004). However, there was no information presented about the effects of the non-metabolized components which retain biologic activity.

The effects of Ginseng and its derivatives on the adaptive immune system have also been widely studied in various experimental models. In many of these studies, Ginseng is touted as possessing adjuvant effects, as there is an enhancement of the humoral response in animals immunized with ovalbumin and dosed with Ginseng (Qin et al., 2006; Sun et al., 2005; Sun et al., 2007; Yang et al., 2007b). This response was measured by analysis of antigen-specific antibody titers. In short-term (10-50 μg on days 1 and 15) treatment of immunized mice with Ginseng/ginsenosides there was an enhanced Th1 and Th2 cytokine production, while long-term (30 consecutive days with 2 g/kg of a 50% ethanol extract of ginseng root) exposure resulted in decreased amounts of cytokines (Liou et al., 2005; Yang et al., 2007a; Yu et al., 2004). Interestingly, in another experiment determining the actions of individual ginsenoside on T cell proliferation induced by

various T cell mitogens, ginsenoside Rb1 and Re enhanced proliferation while Rb2 inhibited proliferation *in vitro* (Cho et al., 2002b). This suggests that ginsenosides can differentially regulate lymphocyte proliferation.

The effects of Ginseng and its components can therefore be described as being diverse. Properties of Ginseng are dependent on the branch of immunity studied, with most studies suggesting decreased inflammatory responses and modulation of adaptive immunity. Additionally, Ginseng possesses different ginsenoside and polysaccharide components that vary with season, time of harvest and location of growth. The composition of these components in Ginseng species may dictate the observed effects on immunity.

Exploiting the effects of notoginseng on APCs for immunotherapy

The information described in the previous section demonstrates that Ginseng possesses immunomodulatory capability. Moreover, as described previously, many of the effects of Ginseng on immune cells occur through the action of ginsenosides. Of the three most popular Ginseng species, the highest concentration of ginsenosides is present in *Panax notoginseng*. Therefore, this herb is ideal for studying the effects of Ginseng extracts on immune cells.

As previously mentioned, APCs are among the first cells present at the site of an infection, releasing cytokines and other inflammatory proteins to combat pathogens. In addition, APCs are initiators and modulators of the adaptive immune response (Banchereau and Steinman, 1998; Medzhitov and Janeway, 1998). They are important intermediaries between microorganisms and the control of the immune system (Lee and

Iwasaki, 2007; Steinman, 2001). For this reason, regulation of APCs should significantly influence both innate and adaptive immunity.

Of the three types of APCs described above, macrophages and DCs are most likely to elicit an adaptive immune response (St Clair et al., 2007). This is because they reside in areas more likely to be exposed to antigens where they can phagocytose pathogens. As a result of their superior antigen presenting capabilities, DCs have received the most attention as activators of T cells. Recently, research efforts have been focused on ways to manipulate DCs to control immune function. These studies take advantage of the basic properties of DCs and how they can manipulate them to treat or cure diseases. This includes loading DCs with a particular antigen, as in the case for vaccines against various pathogens such as HIV or even cancer cells. In this case, DCs will direct adaptive immune cells to elicit a response against pathogens or cells bearing this antigen. Alternatively, if suppression of the immune system is desired as in the case of inflammatory or autoimmune diseases, DCs can be manipulated to decrease their innate and adaptive immune functions. This may include reducing TLR activation, decreasing the production of pro-inflammatory cytokines, increasing the production of anti-inflammatory cytokines, downregulating accessory/costimulatory molecules and suppression of T cell responses (Morel et al., 2003).

Various strategies have been used, including treatment with pharmaceutical non-steroidal and steroidal agents, to manipulate APC activity. However, as numerous people suffer from inflammatory diseases, research continues on finding agents for modulating the immune system as a remedy for these ailments.

Panax notoginseng has been demonstrated to have numerous effects on the immune system, including decreasing inflammation. However, further research is necessary to elucidate the effects of this herbal on both innate and adaptive immunity. As APCs play a major role in coordinating events in both innate and adaptive immune function, studying the effects of notoginseng on the fate and function of APCs should provide benefit to the fields of immunopharmacology and biomedical science.

Hypothesis

Panax notoginseng alters APC fate and function by decreasing activation molecule expression and inflammatory mediator production by macrophages and DCs. To address this hypothesis, several studies were performed focusing on the following specific Aims:

Aim 1. (Chapter 2)

To determine if *Panax notoginseng* attenuates LPS-induced pro-inflammatory mediators in macrophages using RAW264.7 cells.

Aim 2. (Chapter 3)

To investigate whether *Panax notoginseng* decreases APC activation induced by selected Toll-Like receptor ligands in murine DC2.4 dendritic cells *in vitro*.

Aim 3. (Chapter 4)

To evaluate the effects of *Panax notoginseng* on innate and adaptive immune fate and function of primary APCs *in vitro* and *in vivo*.

In addition to these three specific Aims, studies were also conducted to determine whether *Panax notoginseng* could be used as a therapeutic agent to treat diseases with an inflammatory component. In our first set of studies, the general toxicity of ginseng was investigated with specific attention to the immune system. The second study was based on our previous results on the effects of notoginseng on decreased antigen uptake and inflammatory mediator production by APCs. In these experiments we assessed the effects of notoginseng on uptake of acetylated LDL (ac-LDL) by BMDCs. The aims for these two studies are described below.

Aim 4. (Chapter 5)

To determine the immunotoxicological effects of *Panax notoginseng in vivo* in ova immunized Balb/c mice.

Aim 5. (Chapter 6)

To investigate whether *Panax notoginseng* can reduce uptake of ac-LDL by BMDCs.

Finally, Chapter 7 will include a summary of the results presented in this dissertation and the significance and long-term implications of this study. This chapter will also provide insight on the future directions for continued research on the effects of *Panax notoginseng* on immune cells.

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CHAPTER 2

Panax Notoginseng Attenuates LPS-Induced Pro-Inflammatory Mediators in RAW264.7 cells.

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Abstract

Herbals or dietary supplements are not regulated as drugs by the United States Food and Drug Administration (USFDA) although many may have associated therapeutic effects and toxicities. Therefore, the immunomodulatory effects of the herbal extract *Panax notoginseng* on cultured macrophages (RAW264.7 cells) were investigated to address potential therapeutic or toxic effects. Cells were stimulated with LPS (1 $\mu\text{g/ml}$) and treated with notoginseng at 5, 25 and 50 $\mu\text{g/ml}$. Notoginseng inhibited the LPS-induced production of TNF- α and IL-6 by the cultured macrophages in a concentration-dependent manner. The expression of COX-2 and IL-1 β mRNA was also attenuated by notoginseng. TNF- α production was inhibited in samples treated with notoginseng 24 hours before, or at the same time as LPS stimulation, but not in samples treated 8 hours after LPS stimulation. Notoginseng reduced expression of the accessory molecules CD40 and CD86 on the RAW264.7 cells while CD14 and TLR4 expression remained unaffected. Furthermore, Rb1 and Rg1 ginsenosides also inhibited macrophage production of TNF- α , but to a lesser extent than did the whole notoginseng extract. Collectively, these results indicate that notoginseng inhibits LPS-induced activation of RAW264.7 macrophages and demonstrates that notoginseng possesses anti-inflammatory and immunosuppressive properties *in vitro*.

Keywords: *Panax notoginseng*; Ginsenosides; RAW264.7; Macrophages; Inflammation.

Abbreviations: LPS, lipopolysaccharide; TNF- α , tumor necrosis factor-alpha; IL-1 β , Interleukin-1 beta; IL-6, Interleukin-6; COX-2, cyclooxygenase-2.

Introduction

Over the last decade there has been a steady increase in the use of herbal and dietary supplements. The rise in popularity of natural products may be attributed to more aggressive sales tactics, enhanced Internet accessibility and dissatisfaction with conventional medicines (Barnes *et al.*, 2004). There also exists a misconception that “natural” is synonymous with safe despite the fact that herbal products can cause medical problems if not taken correctly or if taken in excessive amounts (Klepser Bailey and Micheal, 1999). The United States Food and Drug Administration (USFDA) does not classify natural products as drugs despite associations with both therapeutic effects and toxicities. This means that, unlike conventional drugs, natural products are not required to meet rigorous standards to demonstrate safety, efficacy and mechanisms of action. Recent evidence demonstrates that natural products have the capacity to interact with conventional drugs via modulation of various xenobiotic metabolizing enzymes such as CYP2C9 and CY3A4 (He and Edeki, 2004). The rise in natural product usage and the potential for adverse or advantageous reactions has led to increased attention to their potential safety and efficacy.

Among these widely used natural products, ginseng was the second most frequently purchased herbal by the US adult population in 2002 (Barnes *et al.*, 2004). Over thirteen species of ginseng have been identified, including *Panax notoginseng* (Burk.) F.H. Chen ex C.Y. Wu & K.M. Feng (Yun, 2001). The biologically active compounds of ginseng species are thought to be the saponins of which the ginsenosides Rb1, Rg1, Re1, Rh1 and the notoginsenoside R1 are considered to be the major components of *Panax notoginseng*

(Li *et al.*, 2005; Yun, 2001). The biological activity of *Panax notoginseng* is similar to the more widely known *Panax ginseng* plant, with differences in activity associated with higher levels of ginsenosides in the notoginseng species (Chuang *et al.*, 1995; Zhu *et al.*, 2004). Ginseng is highly regarded in China for its therapeutic ability to stop hemorrhages, influence circulation, act as a tonic, induce variable effects on systemic blood pressure, and generate analgesic and anti-inflammatory effects (Xu *et al.*, 2003). Notoginseng's cardiovascular effects occur via inhibition of calcium entry through receptor-mediated calcium channels without affecting voltage gated calcium channels or intracellular calcium release (Kwan, 1995a). The ginsenosides Rb1 and Rg1 also have stimulatory effects on the central nervous system. They can improve memory, learning and confer neuroprotection in some instances (Attele *et al.*, 1999).

The immunomodulatory effects of notoginseng have not been fully characterized. Several studies have described the effects of ginseng and ginsenosides on the immune system, but specific mechanisms of action have yet to be identified. Noted immunologic effects include anti-allergic and anti-inflammatory activities of Rh1 (Park *et al.*, 2004), a reduction in TNF- α levels by Rb1 (Smolinski and Pestka, 2003a), an increase in both humoral and cell-mediated immune responses by Rg1 (Kwan, 1995a), and a decrease in phospholipase 2 activity and neutrophil numbers by *P. notoginseng* extract (Li and Chu, 1999).

Macrophages are immune cells usually dispersed throughout the body. They are particularly important in innate immunity as they are among the first cells responding to microbial infection. They can kill pathogens directly by phagocytosis and indirectly via the secretion of pro-inflammatory cytokines such as the TNF- α , IL-1 β , and IL-6. These

cytokines lead to a variety of responses including the induction of cyclooxygenase-2 (COX-2) expression (Turini and DuBois, 2002), increased expression of adhesion molecules on vascular endothelial cells (Luscinskas and Gimbrone, 1996), the induction of acute-phase response proteins by the liver (Diehl and Rincon, 2002), and the production of colony stimulating factors by activated endothelial cells which induce hematopoiesis (Watowich, 1996).

Macrophages also serve an important role as an interface between innate and adaptive immunity. They are responsible for processes such as antigen processing and presentation to antigen-specific T cells. Following activation, macrophages can modulate expression of accessory molecules such as CD14 and TLR4 that facilitate LPS uptake and signaling (Dunzendorfer *et al.*, 2004). Activation also induces costimulatory molecules such as CD40 and CD86 that promote sustained stimulatory interactions with T cells and the generation of adaptive immunity (Grewal and Flavell, 1998; Lenschow *et al.*, 1996). Any compound capable of modulating macrophage activation and/or function holds great promise for use in the treatment of chronic inflammatory diseases such as asthma, atherosclerosis and rheumatoid arthritis.

In this study, we hypothesized that notoginseng would reduce the production of pro-inflammatory mediators by LPS-stimulated macrophages. To test this hypothesis, the production of the inflammatory mediators, TNF- α and IL-6, as well as the expression of IL-1 β and COX-2 mRNA were evaluated in RAW264.7 murine macrophages treated with LPS and notoginseng. The effect of notoginseng on the expression of accessory molecules CD14, TLR4, CD86 and CD40 on RAW264.7 cells was also studied. Further studies examined the effects of the purified ginsenosides, Rb1 and Rg1, on LPS-induced

TNF- α and IL-6 production. Our results confirm and extend previous findings of the immunomodulatory effects of notoginseng. Experiments in this paper demonstrate for the first time that these effects are dependent on the duration of treatment, can alter pro-inflammatory molecule expression at the mRNA level, and can modulate important accessory molecules commonly expressed on macrophages. Moreover, these results demonstrate that notoginseng attenuates the production of several pro-inflammatory mediators by macrophages following *in vitro* stimulation by LPS.

Materials and Methods

Chemicals

Noto-GTM extracts from the plant *Panax notoginseng* (Burk.) F.H. Chen ex C.Y. Wu & K.M. Feng were kindly supplied by Technical Sourcing International, Inc. (TSI) (Missoula, MT). Notoginseng was extracted from the root of the plant using ethanol and standardized to contain Rb1 and Rg1 ginsenosides at 35 and 34% of the whole extract, respectively. The quantification of Rb1 and Rg1 in the notoginseng extract was determined by high-performance liquid chromatography analysis by TSI. Documentation by TSI also showed no detectable levels of *Escherichia coli* (E. coli) or *Salmonella enterica* in the notoginseng preparation (unpublished data). Certification of analyses were approved by Xia Ronglong (QA manager TSI). The extract was dissolved in complete media (see below) or culture-grade DMSO (Sigma-Aldrich, St. Louis, MO) and subsequently sterile-filtered through a 0.22 μ M Millipore membrane. The purified ginsenosides Rb1 and Rg1 were purchased from Indofine Chemical Company, Inc.

(Hillsborough, NJ). Lipopolysaccharide (LPS) from *E. coli* (055:B5) was obtained from Sigma-Aldrich.

Cell Culture

RAW264.7 cells were obtained from ATCC (Manassas, VA). Cells were grown in RPMI (GibcoBRL, Grand Island, N.Y), supplemented with 10% FBS (Hyclone, Logan, UT), 50 μ M mercaptoethanol, 20 mM HEPES, 10 mM sodium pyruvate and 50 μ g/ml gentamicin (GibcoBRL, Grand Island, N.Y). RAW264.7 cells were maintained via weekly passage and cells were utilized for experimentation at 60-80% confluency.

Cell Activation and Treatment

RAW264.7 cells (5×10^5 cells per well) were incubated overnight at 37 $^{\circ}$ C and 5% CO₂ in 6-well plates to facilitate attachment and spreading before experimentation. Cells were then stimulated with 1 μ g/ml LPS and treated with 0, 5, 25 or 50 μ g/ml notoginseng or Rb1 and Rg1 ginsenosides at concentrations equivalent to that in 50 μ g/ml notoginseng. After an additional 24 hours supernatants were collected for evaluation by ELISA and cells harvested for RT-PCR and FACS analysis.

Cytokine Assays

The inhibitory effects of notoginseng on the production of IL-6 and TNF- α were measured by enzyme-linked immunosorbent assay (ELISA) using supernatants collected from treated cells. Samples were analyzed per the manufacturer's recommendations with mouse cytokine-specific BD OptEIA ELISA kits (BD PharMingen, San Diego, CA).

RT-PCR

RT-PCR for the detection of IL-1 β and COX-2 mRNA was performed as previously described (Shepherd *et al.*, 2001). Briefly, total RNA was isolated using Trizol and reverse transcribed into cDNA. COX-2 and IL-1 β transcripts were identified using specific forward and reverse primers as per manufacturer's instructions (Clontech, Palo Alto, CA). β -2 microglobulin expression was included as an internal, housekeeping gene control. Ethidium bromide-stained reaction products were separated by electrophoresis on a 2% agarose gel in TBE and visualized by UV transillumination. Images were captured by a Kodak EDAS 290 camera system (Kodak, Rochester, NY).

The primers used in these experiments were designed to span introns thereby allowing differentiation between amplified genomic DNA and cDNA PCR products. Primers sequences used were β -2 microglobulin 5' ATGGCTCGCTCGGTGACCCT and 3' TCATGATGCTTGATCACATG, IL-1 β 5' ATGGCAACTGTTCTGAACTCAACT and 3' CAGGACAGGTATAGATTCTTTCCTTT and COX-2 5' AACACAGCTACGAAAACC and 3' CACAGTATGATGTAACAGTCC.

Flow cytometry

The detection of accessory molecule expression on RAW264.7 cells by fluorescent activated cell sorting (FACS) analysis was performed as previously described (Shepherd *et al.*, 2001). Briefly, RAW264.7 cells were harvested and washed with PAB (1% bovine serum albumin and 0.1% sodium azide in PBS). Cells were blocked with 50 μ l of 600

µg/ml purified rat and/or hamster IgG (Jackson ImmunoResearch, West Grove, PA) for 10 minutes to inhibit non-specific staining. Fluorochrome-conjugated antibodies to mouse were then added at 1 µg/ml concentrations for an additional 10 minutes. The antibodies used in these experiments were CD86-APC, CD40-PE, TLR4-PE, CD14-FITC, and their corresponding isotype controls (BDPharmingen, San Diego, CA, except for the anti-TLR4 eBiosciences, San Diego, CA). One hundred thousand viable cells per treatment (as determined by light scatter profiles and propidium iodide staining) were analyzed using a BD FACSAria flow cytometer and FACSDiva software (BD Biosciences, San Jose, CA).

Statistics

All statistical analyses were performed using GraphPad Prism 4.0a for the Macintosh (GraphPad Software, San Diego, CA). Data signify the mean +/- of 3 samples and are representative of 3 independent experiments. Differences between two means were analyzed by Student's t-test. Data sets with multiple comparisons were evaluated by one-way analysis of variance (ANOVA) with Dunnett's post test. Values of $p < 0.05$ were determined to be significant.

Results

Notoginseng suppresses the LPS-induced production of TNF- α and IL-6 by RAW264.7 cells

RAW264.7 cells are an immortalized monocyte/macrophage murine cell line that has been used extensively to evaluate monocyte and macrophage fate and function *in vitro*.

To evaluate the potential effects of notoginseng on macrophage function, RAW264.7 cells were stimulated with 1 μ g/ml LPS and concomitantly treated with notoginseng. Unstimulated RAW264.7 cells secrete a basal level of TNF- α but barely detectable amounts of IL-6 (Figs. 2-1A and 2-1B, respectively). At the highest concentration tested, notoginseng did not evoke TNF- α or IL-6 release in the absence of LPS stimulation. The addition of LPS resulted in a 9-fold and 120-fold increase in TNF- α and IL-6 protein levels, respectively. Notoginseng significantly inhibited the production of both TNF- α and IL-6 in a concentration-dependent manner. At the highest concentration of notoginseng tested, TNF- α production was reduced approximately 3-fold while IL-6 production was reduced 7-fold. Importantly, no cytotoxicity was observed at any of the concentrations of notoginseng examined, as assessed by trypan blue exclusion (data not shown). Also, no significant differences were observed in cell recoveries between notoginseng- and control-treated cells.

COX-2 and IL-1 β mRNA levels are decreased in LPS-stimulated RAW264.7 cells following exposure to notoginseng

Because of the prominent role of COX-2 and IL-1 β in the inflammatory response, the potential for notoginseng to alter the expression of these mediators was investigated. COX-2 and IL-1 β mRNA levels in RAW264.7 cells were evaluated by RT-PCR. The mRNA expression of the housekeeping gene β -2 microglobulin was used to normalize samples. Untreated RAW264.7 cells expressed detectable levels of both COX-2 and IL-1 β mRNA that increased following LPS stimulation (Fig. 2-2). Notoginseng inhibited

Figure 2-1

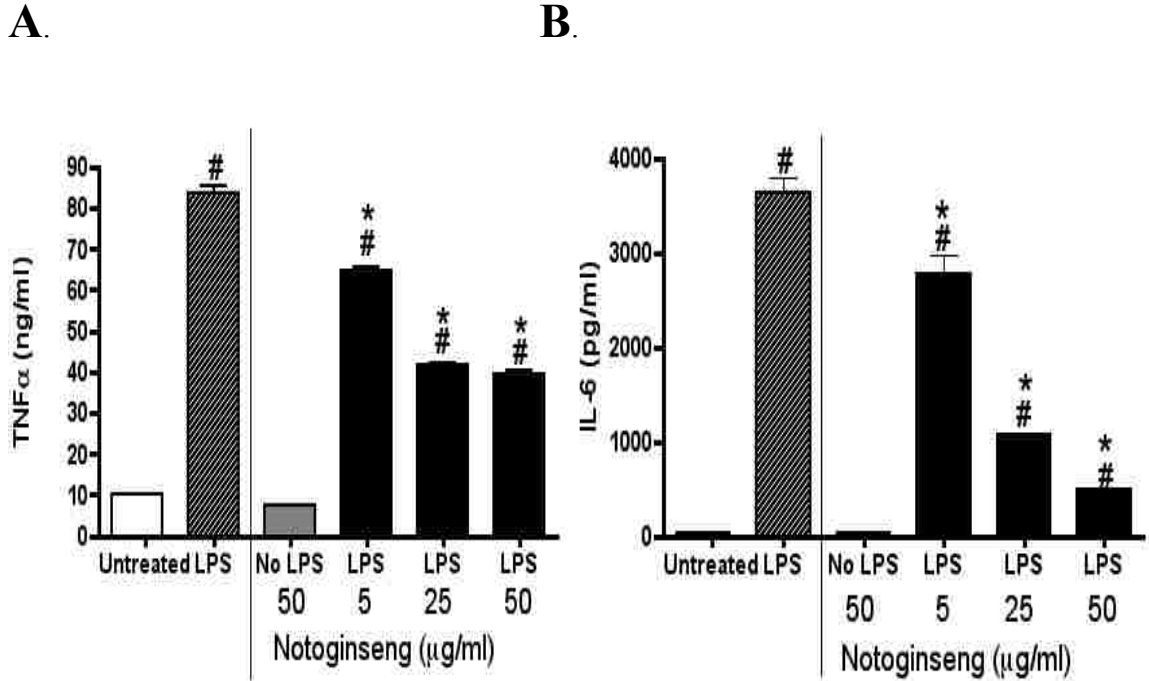


Figure 2-1. Notoginseng inhibits the LPS-induced TNF- α and IL-6 production by RAW 264.7 cells. Cells were treated simultaneously with 1 μ g/ml LPS and 5, 25 or 50 μ g/ml of notoginseng. Supernatants were collected after 24 hours and assayed for TNF- α (A) and IL-6 (B) production as described in the *Materials and Methods*. Data represents mean \pm SEM of 3 samples. # indicates significant difference between stimulated and unstimulated cells; * indicates significant differences between the LPS-stimulated control- and notoginseng-treated samples ($p < 0.05$). Data are representative of 3 independent experiments.

Figure 2-2

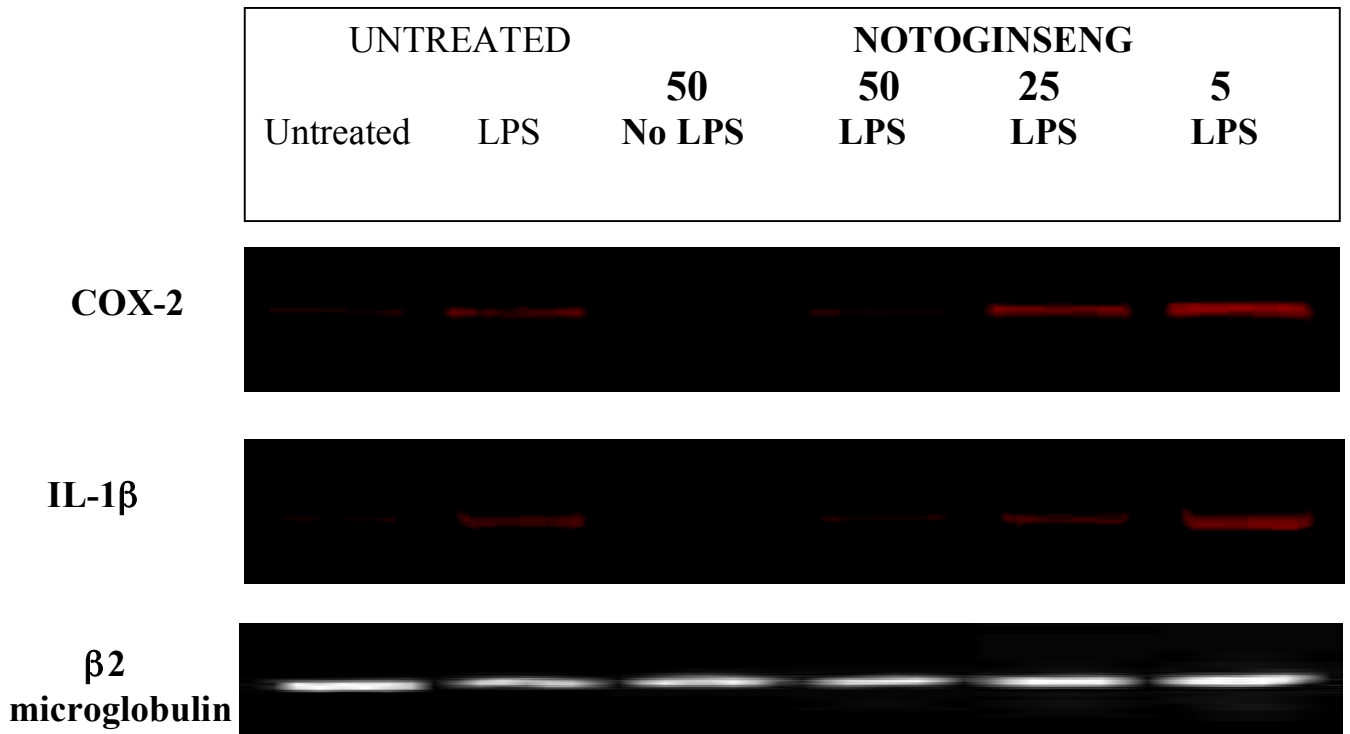


Figure 2-2. Notoginseng decreases COX-2 and IL-1 β mRNA levels following LPS activation of cultured macrophages. RAW 264.7 cells were treated simultaneously with 1 μ g/ml LPS and 5, 25 or 50 μ g/ml of notoginseng for 24 hours. Cells were harvested, and mRNA extracted and transcribed into cDNA. Expression levels of COX-2 and IL-1 β mRNA were measured by RT-PCR and visualized by ethidium bromide staining of a 2% agarose gel. β -2 microglobulin (β 2) was included as a housekeeping gene to normalize all samples. Data are representative of 3 separate experiments.

LPS-induced IL-1 β mRNA expression in a concentration-dependent manner and reduced the expression of COX-2 mRNA in LPS-stimulated macrophages at the higher concentrations examined (Fig. 2-2). Interestingly, mRNA expression levels of both pro-inflammatory genes were decreased in RAW264.7 cells that were not stimulated with LPS, but treated with the highest concentration of notoginseng only.

Notoginseng selectively modulates the expression of key accessory molecules

The expression levels of accessory/costimulatory molecules on macrophages can dually affect their inflammatory responsiveness and capacity to function as antigen presenting cells. To determine if notoginseng modulates accessory molecule expression on activated macrophages, RAW264.7 cells were treated concomitantly with LPS and 50 μ g/ml of notoginseng and the fluorescence intensity of CD40, CD86, CD14 and TLR4 measured by flow cytometry. Only the highest concentration of notoginseng was evaluated in this experiment since significant inhibition of other inflammatory mediators had been demonstrated at this concentration (Figs. 2-1 and 2-2). Stimulation of RAW264.7 cells with LPS increased the expression of CD40, CD86 and CD14 (Table 2-1). Conversely, LPS treatment results in down-regulation of TLR4 expression on RAW264.7 cells as has been previously reported (Akashi *et al.*, 2000). Notoginseng decreased the LPS-induced expression of CD40 by almost 20% (Fig. 2-3A) and CD86 by 30% (Fig. 2-3B) on RAW264.7 cells. In contrast, cell surface expression of TLR4 and CD14 remained unchanged following notoginseng exposure (Table 2-1).

Table 2-1. Notoginseng differentially affects the expression of LPS-induced accessory molecules.

	CD40 MCF^b	CD86 MCF	CD14 MCF	TLR4 MCF
Untreated^a	320 ± 16	538 ± 25	117 ± 9	354 ± 25
+ LPS	513 ± 21 ^c	1060 ± 25 [#]	192 ± 8 [#]	252 ± 23 [#]
+ LPS NOTOGINSENG	429 ± 31 ^{#*}	737 ± 35 ^{#*}	194 ± 4 [#]	244 ± 21 [#]

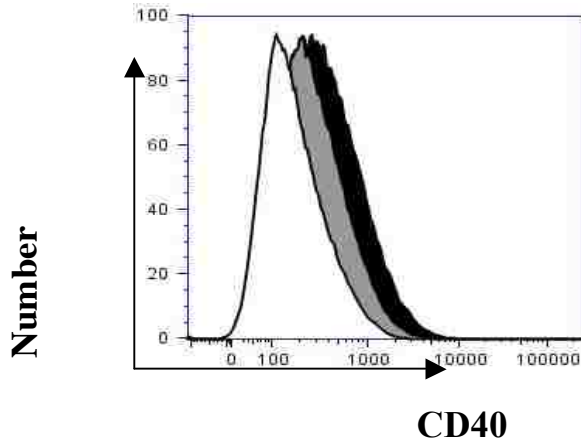
^a RAW264.7 cells were either unstimulated or stimulated with LPS (1µg/ml). Some of the LPS-treated samples were concomitantly treated with notoginseng (50 µg/ml) for 24 hours.

^b Mean Channel Fluorescence (MCF) was determined by flow cytometry.

^c Data shown are representative of four independent experiments. Error bars indicate mean ± SEM; #, $p < 0.05$ for the comparison of unstimulated and LPS-activated cells; *, $p < 0.05$ for the comparison of LPS-stimulated cells treated with notoginseng or left untreated.

Figure 2-3

A.



B.

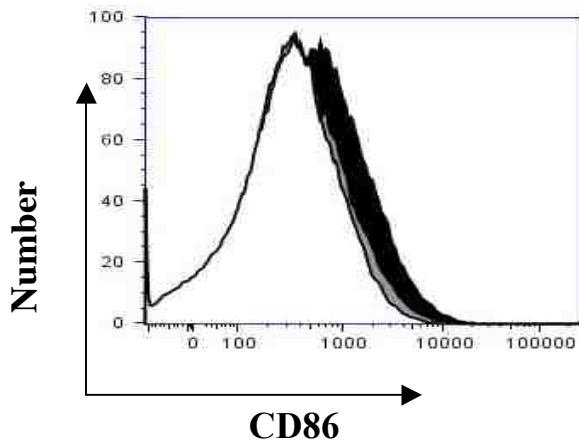


Figure 2-3. Notoginseng treatment of RAW264.7 cells selectively modulates cell surface expression of accessory molecules. RAW264.7 cells were unstimulated (white histogram), stimulated with LPS (black histogram) or stimulated with LPS and concomitantly treated with 50 $\mu\text{g/ml}$ notoginseng (grey histogram) for 24 hours. Representative histograms demonstrating the cell surface expression of CD40 (A) and CD86 expression (B) on viable RAW264.7 cells receiving various treatments were determined by FACS analysis. Values in histograms correspond to specific Mean Channel Fluorescence (MCF) values for each treatment. Data are representative of 3 independent experiments.

TNF- α production is affected by notoginseng in a time-dependent manner

To assess if the duration of notoginseng exposure results in differential effects on TNF- α production, RAW264.7 cells were either pretreated with notoginseng for 24 hours prior to LPS addition (-24 hr timepoint), treated with notoginseng and LPS simultaneously (0 hr), or treated with notoginseng 8 hours (+8 hrs) after LPS addition. All samples were collected 24 hours after LPS stimulation. Consistent with the results presented in Fig. 2-1, concomitant treatment of RAW264.7 macrophages with LPS and notoginseng significantly suppressed the production of TNF- α after 24 hours of culture by more than 50% (Fig. 2-4). Pretreatment of RAW264.7 cells with notoginseng for 24 hours resulted in a 17% decrease in TNF- α levels. It should be noted that for these cultures, the herbal extract was not removed from the medium after 24 hours but remained for the entire culture period of 48 hours. In contrast, delaying the addition of notoginseng by 8 hours relative to LPS treatment, resulted in no significant reduction of TNF- α although a trend towards lesser levels was observed.

The purified ginsenosides Rb1 and Rg1 modulate the production of TNF- α by RAW264.7 cells

The whole notoginseng extract used in the preceding experiments is comprised of several ginsenosides although primarily of Rb1 (35%) and Rg1 (34%). Because ginsenosides are believed to comprise much of the biological activity of notoginseng, we sought to define the potential inhibitory effects of the purified ginsenosides Rb1 and Rg1 on LPS-induced TNF- α production by RAW 264.7 cells. To more closely mimic the approximate concentration of ginsenosides present in the whole extract, Rb1 and Rg1 were

Figure 2-4

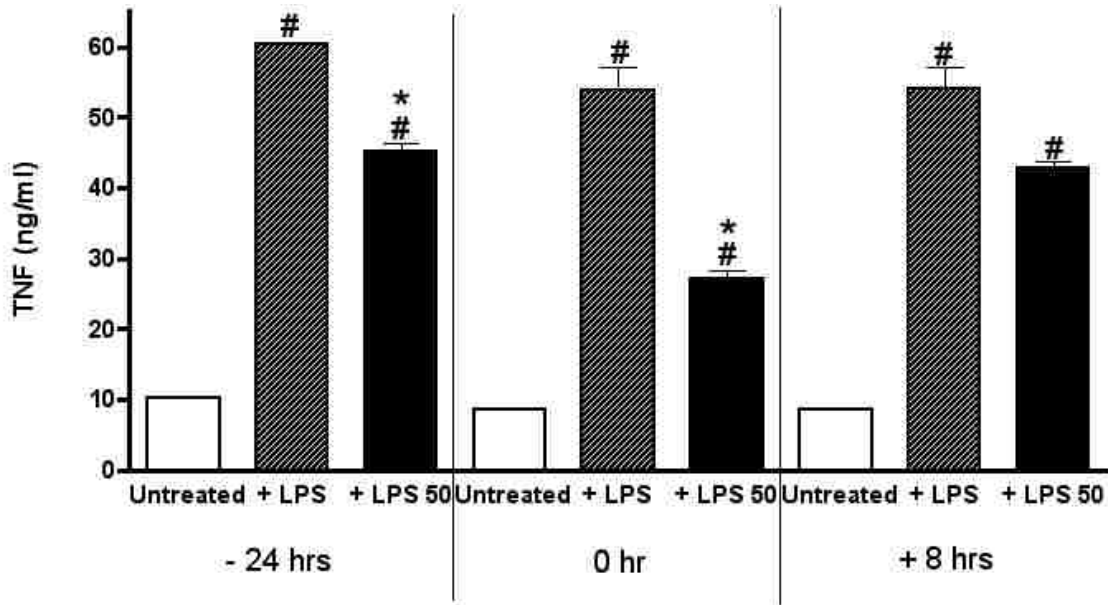


Figure 2-4. Variable notoginseng treatment affects the LPS-induced production of TNF- α by RAW 264.7 cells. Cells were treated with notoginseng at three different timepoints: -24 hours, 0 hours and 8 hours, relative to LPS stimulation. Supernatants were collected 24 hours after LPS addition and TNF- α levels measured by ELISA. Data represents mean \pm SEM of 3 samples. # Indicates significant differences between stimulated and unstimulated cells; * indicates significant differences between LPS-stimulated controls and LPS-stimulated, notoginseng-treated samples ($p < 0.05$). Data are representative of 3 separate experiments.

standardized to approximate concentrations found in 50 µg/ml of the intact notoginseng extract. Following LPS activation of RAW264.7 cells, both Rb1 and Rg1 and a combination of the Rb1/Rg1 purified compounds significantly inhibited TNF- α production (Fig. 2-5A). However, neither ginsenoside alone or in combination attenuated TNF- α production to the extent that was observed following exposure to the whole notoginseng extract. In addition, the whole notoginseng extract and ginsenoside Rg1 caused significant inhibition of LPS-induced IL-6 by RAW264.7 cells (76% and 24% of LPS-stimulated controls, respectively). Conversely, no significant suppression was detected after the addition of Rb1 to these cultures (Fig. 2-5B).

Discussion and conclusion

In the current study, the potential for notoginseng to decrease the LPS-induced production or expression of cytokines, TNF- α , IL-6, and IL-1 β , was tested because of their significance in inflammatory conditions. The production of these inflammatory molecules by RAW264.7 cells can be induced in response to LPS stimulation (Beutler, 2003; Rice and Bernard, 2005). TNF- α is primarily produced by monocytes, macrophages and T-cells (Bondeson, 1997) and has various pro-inflammatory effects on many cell types. It is a potent activator of macrophages, can stimulate the production or expression of IL-1 β , IL-6, prostaglandin E₂, collagenase, type I and III collagens, adhesion molecules and is a growth factor for both B and T lymphocytes (Bondeson, 1997). Both TNF- α and IL-1 β can lead to cartilage destruction and bone resorption and are important cytokines in chronic inflammatory diseases such as rheumatoid arthritis (RA) (Bondeson, 1997; Dayer, 2004). IL-6 has also been shown to be important in a

Figure 2-5

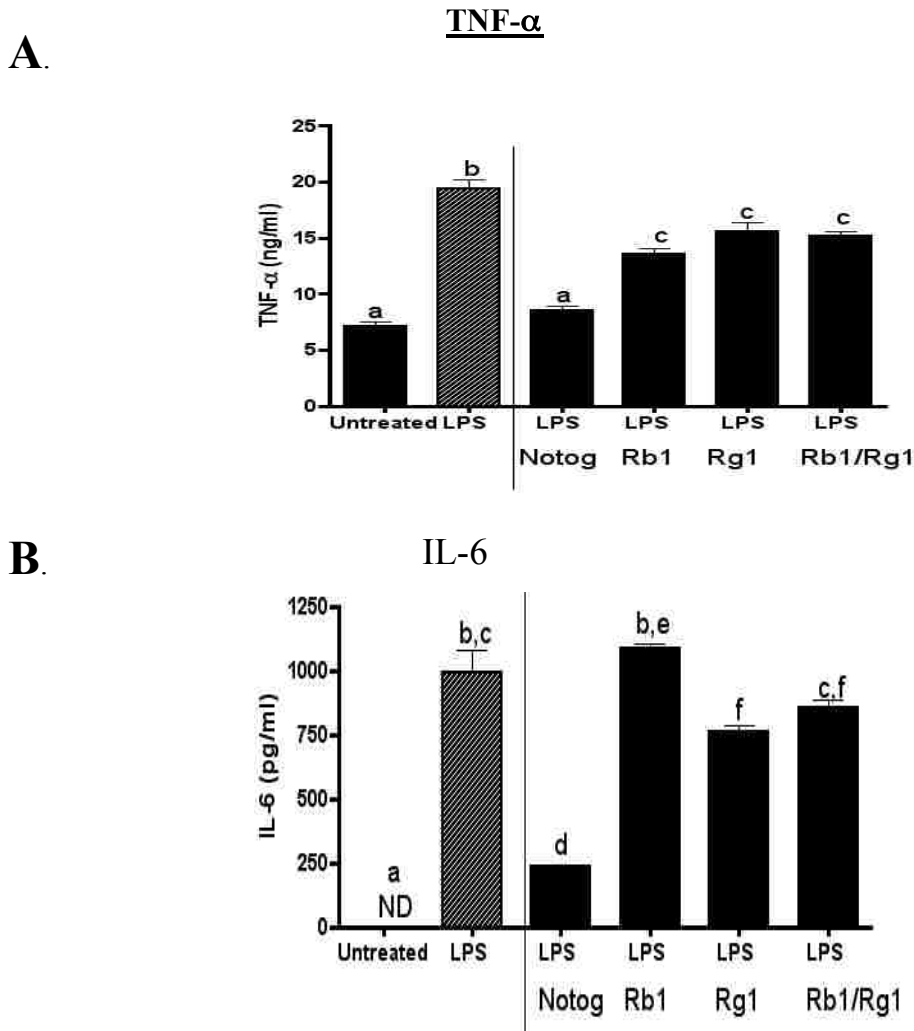


Figure 2-5. The production of TNF- α and IL-6 is selectively suppressed by the whole notoginseng extract and purified ginsenosides. LPS-stimulated cells were treated with 50 μ g/ml whole notoginseng extract or purified Rb1 and/or Rg1 compounds. Supernatants were collected after 24 hours and assayed for TNF- α and IL-6 production by ELISA. Data represents mean \pm SEM of 3 samples. Statistically significant differences ($p < 0.05$) between treatment groups are indicated by different letters. Data are representative of 3 independent experiments.

variety of inflammatory conditions and is particularly important in the production of acute phase proteins (Diehl and Rincon, 2002). Although the present study models predominately an acute inflammatory condition, our results provide valuable information variety of inflammatory conditions and is particularly important in the production of on the effects of notoginseng on macrophages, cells critical in the process of chronic inflammation (Lefkowitz *et al.*, 1995). The results presented herein show that notoginseng can decrease the LPS induced production of TNF- α and IL-6 and reduce the mRNA expression of IL-1 β and COX-2 in LPS-stimulated RAW264.7 cells (Figs. 2-1 and 2-2). Cyclooxygenase is a key enzyme involved in the conversion of arachidonic acid into prostaglandins (Turini and DuBois, 2002). It has been demonstrated to be a critical pro-inflammatory enzyme contributing to the development of many chronic inflammatory diseases such as cardiovascular disease, cancer and RA (Rocca and FitzGerald, 2002). IL-1 β is a cytokine that is produced early in the generation of an inflammatory response (Stylianou and Saklatvala, 1998). It is produced in increasing amounts in diseases such as RA and atherosclerosis (Andreaskos *et al.*, 2004a; Bondeson, 1997). Our results demonstrate that notoginseng effectively inhibits the generation of cytokines and enzymes in this monocyte/macrophage cell line that are paramount in the generation of an inflammatory response.

In addition to the effects on cytokines production, notoginseng also significantly affects the expression of key accessory molecules expressed on macrophages. Bacterial products are potent inducers of many pro-inflammatory genes including accessory/costimulatory molecules such as CD40 and CD86. CD40 and CD86 subsequently promote sustained interactions between APCs and T cells (Grewal and

Flavell, 1998). When stimulated with LPS, the cell surface expression of CD86 and CD40 is increased on RAW264.7 cells (Fig. 3 and Table 1). The upregulation of these molecules is typically observed during the course of an inflammatory response and functions to facilitate the recruitment and activation of leukocytes (Grewal and Flavell, 1998; Lenschow et al., 1996; Li and Stark, 2002). Notoginseng effectively decreased the expression of CD40 and CD86 molecules (Fig. 3). Because CD40 and CD86 are required for productive interactions between T cells and antigen presenting cells, decreasing their expression would be expected to attenuate conditions such as chronic inflammatory disease and autoimmunity (Lenschow et al., 1996; Quezida et al., 2004). In our experiments, LPS was used as the prototypical inflammatory stimulus because of its ability to initiate a range of pro-inflammatory mediators (Abreu and Arditi, 2004). LPS signaling occurs when LPS binding protein catalyzes the transfer of LPS to membrane or soluble CD14, which then mediates recognition of LPS via TLR4 signaling (Dunzendorfer et al., 2004). LPS (as well as TNF- α and IL-1 β) is an effective activator of NF- κ B (Hanada and Yoshimura, 2002; Karin and Ben-Neriah, 2000a). NF- κ B activation induces the expression of many inflammatory cytokine genes (including TNF- α and IL-1 β) and accessory molecules. In our studies, notoginseng did not affect the expression of TLR4 or CD14 on RAW264.7 cells suggesting that this herbal extract does not decrease LPS uptake or TLR 4 signaling in these cells. However, although TLR4 and CD14 expression was unaffected by notoginseng, LPS signaling could potentially be interrupted in its downstream signaling pathway as has been previously reported (Lee et al., 2002). In these studies, disruption of NF- κ B activation was demonstrated in both mouse skin cells and the human pro-myelomonocytic cell line, HL-60, by purified ginsenosides (Keum et

al., 2003a; Lee et al., 2002). Furthermore, Oh et al. recently reported that 20(S)-protopanaxatriol, a ginsenoside metabolite, inhibits iNOS and COX-2 expression via the inactivation of NF- κ B in LPS-stimulated RAW264.7 cells (Oh et al., 2004b). Currently, studies in our laboratory are underway to further characterize the effects of notoginseng on the activation of NF- κ B.

Varying the duration of notoginseng exposure results in differential effects on the inflammatory responsiveness of our cultured macrophages. In studying the time-dependent effects of notoginseng on TNF- α production, a trend was observed towards decreasing LPS-induced production of TNF- α by cells stimulated 8 hours prior to notoginseng addition. However, significant suppression of this cytokine was only noted for both the pretreated and concomitant-treated samples (Fig. 2-4). Similar results in our laboratory have also been observed with the dendritic cell line, DC2.4 (manuscript in preparation). Reduced effects following delayed notoginseng treatment may be attributed to an inability of notoginseng to disrupt LPS-stimulated signaling cascades following an initial activation period as notoginseng may affect initial events in the activation of RAW264.7 cells. Alternatively, LPS signaling may also reduce the uptake of notoginseng into cells ultimately decreasing its effectiveness. Thus, it is possible that notoginseng and LPS may bind to a similar receptor, thereby antagonizing the uptake of the other, if not added concurrently. This possibility is currently being investigated in our laboratory.

Ginsenosides are believed to be the primary bioactive compounds in notoginseng. Ginsenoside composition of plants can vary widely depending on factors such as time of harvest, location and seasonal variations (Lenschow *et al.*, 1996). These parameters can result in the “yin and yang” in ginseng with various extracts containing significantly

different activities based on variable ginsenoside profiles (Sengupta et al., 2004). Therefore, from a clinical perspective it is important to determine if a particular ginsenoside accounted for the bioactivity of notoginseng. To address this possibility, purified samples of ginsenosides Rb1 and Rg1 were obtained and their concentration matched to that present in our whole notoginseng extract. Although these purified ginsenosides suppressed TNF- α production, the degree of suppression was not as great as that observed in our original notoginseng samples even when both ginsenosides were combined (Fig. 2-5). This result could be because although Rb1 and Rg1 accounted for approximately 70% of our extract, the ginsenoside Re1 and notoginsenoside R1 (each comprising approximately 4% of notoginseng) may also be contributing to the activity of the whole extract. Indeed, studies have shown that notoginsenoside R1 can significantly antagonize the endotoxin-induced activation of endothelial cells *in vitro* and endotoxin-induced lethality in mice (Zhang et al., 1997). Furthermore, our whole notoginseng extract was obtained from a different commercial source than our purified ginsenosides. Thus, even if these compounds are similar in concentration, stereoisomerism may still exist. Stereoisomerism of natural and synthetic compounds has been shown to contribute to different activities *in vivo* and *in vitro* (Mullenheim et al., 2001; Smith et al., 2005). *In vitro* studies with different enantiomers of lipoic acid showed that the *S* isomer had more activity than the *R* isomer. Similar mechanisms could explain the reduced suppressive effects observed when the RAW264.7 cells were treated with the purified ginsenoside compounds but not the whole extract of notoginseng.

In conclusion, the current study demonstrated that notoginseng treatment of RAW264.7 cells results in a decreased production of the inflammatory cytokines TNF- α

and IL-6, mRNA expression of COX-2 and IL-1 β , and cell surface expression of CD40 and CD86 following LPS stimulation. These results establish that notoginseng has potent anti-inflammatory effects and may hold great promise for use in the treatment of acute and chronic inflammatory diseases in humans.

Acknowledgments

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CHAPTER 3

Toll-like receptor ligand-induced activation of murine DC2.4 cells is attenuated by

Panax notoginseng

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Abstract

Dendritic cells (DCs) play a central role in the regulation of both innate and adaptive immune responses. The medicinal herb, *Panax notoginseng*, has been shown to attenuate the production of pro-inflammatory mediators by innate immune cells. Given the importance of DCs in immunity, we investigated the potential for notoginseng extracts to modulate Toll-like receptor (TLR) ligand-induced activation of cultured DC2.4 cells. Following stimulation with LPS, CpG or poly(I:C) and treatment with 0-50 µg/ml notoginseng extract for 24 hours, DCs were evaluated for various phenotypic and functional readouts. Notoginseng reduced the LPS-, CpG- and poly(I:C)-induced production of TNF- α by DC2.4 cells. Also, IL-6 production by notoginseng-treated cells stimulated with LPS and CpG but not poly(I:C) was reduced when compared to controls. TLR ligand-induced CD40 expression was attenuated by notoginseng. In contrast, notoginseng decreased CD86 levels on DCs activated with LPS and poly(I:C) but not CpG. Inhibition of TNF- α production was time-dependent in LPS-stimulated cells, occurring only with pretreatment or concurrent treatment of notoginseng but not after delayed addition of the herbal extract. Additionally, ginsenoside Rg1 more effectively inhibited LPS-stimulated cytokine production by DC2.4 cells than ginsenoside Rb1. Taken together, these results demonstrate that notoginseng inhibits the production of specific inflammatory molecules and innate immune responsiveness by DCs following TLR activation.

Keywords: *Panax notoginseng*; Ginsenosides; DC2.4; Dendritic cells; LPS; Inflammation; Immunity; Toll-like receptor.

Introduction

Dendritic cells (DCs) are the primary professional antigen presenting cells of the immune system. They have the specialized ability to recognize, capture and process antigen (Ag) in both peripheral blood and tissues (Figdor et al., 2004). Like other immune cells, DCs are capable of recognizing microbial components using pattern-associated molecular patterns (PAMPs) that are common constituents of bacteria and viruses. Many PAMPs bind to specific pattern-recognition receptors on DCs, including Toll-like receptors (TLRs) resulting in immune activation. TLR stimulation leads to up-regulation of inflammatory mediators such as the pro-inflammatory cytokines TNF- α and IL-6. Subsequently, DCs mature and migrate to secondary lymphoid organs, where they interact with naïve T cells and induce antigen-specific immune responses (Geijtenbeek et al., 2004). This process of DC maturation includes the sequential loss of endocytotic/phagocytic receptors, upregulation of costimulatory molecules such as CD40 and CD86, and changes in morphology (Banchereau et al., 2000a).

Toll-like receptors are a major family of cell-bound pattern-recognition receptors that sense infection via recognition of PAMPs and signal DCs for activation. For example, TLR3 recognizes viral double-stranded RNA and induces innate immunity, TLR4 is the primary LPS receptor and TLR9 is the receptor for bacterial DNA (Tsujimoto et al., 2006). Natural and synthetic ligands such as poly(I:C), LPS and CpG have been used extensively to model microbial activation of TLR3, TLR4 and TLR9, respectively. Binding of ligands to these TLRs induces nuclear factor kappa B (NF κ B) activation and subsequent upregulation of numerous immune and inflammatory genes (Banchereau et al., 2000a; Hoshino et al., 2002). Studies suggest modulation of these immune and

inflammatory mediators can regulate the generation, development and progression of several inflammatory diseases including rheumatoid arthritis and atherosclerosis (Sharma and Li, 2006a; Wang et al., 2006a). Because DCs are key regulators of inflammation and immunity, one viable approach for the treatment of inflammatory and autoimmune diseases may be found via the intentional modulation of DC fate and function (Figdor et al., 2004; Pulendran et al., 1997).

In the last decade there has been a dramatic increase in the use of dietary supplements including herbal products. Information about the safety and efficacy of most natural products is vastly inadequate. This insufficiency stems from an unregulated industry, which is in stark contrast to the tightly regulated pharmaceutical industry. Under the Dietary Supplement Health and Education Act of 1994, supplements in the U.S. are not subjected to the same safety requirements that apply to prescription and over-the-counter medications (Berman and Straus, 2004b; Science, 2004). This means that unlike conventional drugs, manufacturers of natural products are not required to conduct rigorous tests to demonstrate safety, efficacy or mechanisms of action (Goldma, 2001; Science, 2004). Recently, a survey by the U.S. National Academy of Sciences showed that a majority of consumers believe that herbs are just as safe, effective and cost-efficient as non-herbal medicines (Klepser and Klepser, 1999). In light of these beliefs and the increased usage of herbals, rigorous investigation is needed to demonstrate safety, mechanisms of action and efficacy of these products (Berman and Straus, 2004a).

Ginseng is a widely consumed medicinal herb, both in the United States and worldwide (Barnes et al., 2004). Over thirteen different species of ginseng have been identified. Within the *Panax* genus, extracts of *Panax notoginseng* typically contain

higher ginsenoside concentrations than formulations of the more widely used *Panax ginseng* (Harkey et al., 2001a). Ginsenoside compounds are saponins which are unique to the *Panax* species and are purported to possess the primary pharmacological activity of ginseng (Harkey et al., 2001a). These compounds are also used as markers for quality control and standardization of *Panax* species. Over twenty different saponins have been identified in notoginseng including ginsenosides, notoginsenosides and gypenoside (Dong et al., 2003b). Of these, the ginsenosides Rg1, Rb1, Rd1 and notoginsenoside R1 are considered the major bioactive components (Dong et al., 2003b).

To date, several studies exist describing the immunomodulatory effects of ginseng and ginsenosides; however, specific mechanisms of action of these phytochemicals remain to be defined. Some of the reported immunologic effects of ginseng and its constituents include the anti-allergic and anti-inflammatory activities of ginsenoside Rh1 (Park et al., 2004), a reduction in TNF- α levels by ginsenoside Rb1 (Smolinski and Pestka, 2003b), an increase in both humoral and cell-mediated immune responses by ginsenoside Rg1 (Kwan, 1995b), and a decrease in phospholipase 2 activity and neutrophil numbers by whole *P. notoginseng* extract (Li and Chu, 1999). Additionally, metabolites of ginseng have been shown to promote human dendritic cell maturation and Th1 polarization *in vitro* (Fogel-Petrovic et al., 2004).

Previously, we have demonstrated that *P. notoginseng* has immunomodulatory effects on murine macrophages *in vitro* (Rhule et al., 2006b). In the present study we hypothesized that notoginseng will reduce the production of inflammatory mediators in TLR ligand-stimulated murine dendritic cells. To test our hypothesis, DC2.4 cells were stimulated with LPS, CpG or poly(I:C) and treated with notoginseng. The production of

pro-inflammatory cytokines as well as the expression of accessory molecules important in DC activation and function were assessed following notoginseng treatment. Concentration- and time-dependent studies were carried out using LPS as the prototypical inflammatory stimulus. Finally, the effects of the purified ginsenosides Rb1 and Rg1 on LPS-induced TNF- α and IL-6 production were compared to our whole notoginseng extract. Our results demonstrate for the first time the immunomodulatory effects of notoginseng and purified ginsenosides on murine DCs in response to several TLR ligands. These results demonstrate that notoginseng selectively attenuates the production of pro-inflammatory mediators by DC2.4 cells following stimulation *in vitro*.

Materials and Methods

Chemicals

NotoGTM extracts from the plant, *Panax notoginseng* (Burk) F. H. Chen ex C.Y. Wu & K.M. Feng were generously provided by Technical Sourcing International, Inc. (TSI, Missoula, MT). Notoginseng was extracted from the root of the plant using ethanol and contained high levels of the ginsenosides Rb1 and Rg1 (35% and 34% of the whole extract, respectively) as determined by HPLC analysis (unpublished data). Notoginseng extracts did not contain detectable levels of *Escherichia coli* or *Salmonella enterica* (unpublished data). Certification of analyses was approved by Xia Ronglong (QA manager, TSI). The extract was dissolved in complete media (see below) or culture-grade DMSO (Sigma-Aldrich, St. Louis, MO) and sterile-filtered through a 0.22 μ M Millipore membrane. The purified ginsenosides Rb1 (CAS number: 41753-43-9) and Rg1 (CAS number: 22427-39-0) were purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ). Lipopolysaccharide (LPS) from *Escherichia coli* (055:B5) was

obtained from Sigma-Aldrich, and CpG oligonucleotide and poly (I:C) were purchased from InvivoGen (San Diego, CA).

Cell Culture

DC2.4 cells, a murine dendritic cell line (Zhenhai et al., 1997), were kindly provided by Dr. Kenneth Rock (University of Massachusetts Medical Center, Worcester, MA). Cells were grown in complete media comprised of DMEM (GibcoBRL, Grand Island, N.Y), supplemented with 10% FBS (Hyclone, Logan, UT), 10 mM HEPES, 2 mM L-glutamine and 50 µg/ml gentamicin (GibcoBRL, Grand Island, N.Y). DC2.4 cells were maintained at 37°C in a humidified incubator with 5% CO₂. Cells were maintained via weekly passage and utilized for experimentation at 60-80% confluency.

Cell Activation and Treatment

DC2.4 cells (1×10^6) were cultured in 1 ml complete media for 24 hrs in 6-well plates. Cells were then stimulated with 1 µg/ml LPS, 0.5 µM CpG or 12.5 µg/ml poly(I:C) and treated with varying concentrations of notoginseng or purified ginsenosides for an additional 24 hours. In some experiments, DC2.4 cells were pre-treated with notoginseng for 24 hrs before LPS stimulation. Supernatants were collected after 24 hours and frozen at -20 °C for subsequent evaluation of cytokines. Additionally, cells were harvested for flow cytometric evaluation of costimulatory molecules.

Cytokine Assays

Levels of IL-6 and TNF-α in supernatants from cultured cells were analyzed by enzyme-linked immunosorbent assay (ELISA). Samples were evaluated per the

manufacturer's recommendations using mouse cytokine-specific BD OptEIA ELISA kits (BD PharMingen, San Diego, CA).

Flow cytometry

The expression of costimulatory molecules on DC2.4 cells was determined by flow cytometric analysis as previously described (Shepherd et al., 2001). Briefly, DC2.4 cells were harvested and washed with PAB (1% bovine serum albumin and 0.1% sodium azide in PBS). Non-specific staining of cells was blocked with 30 µg per sample of purified rat and/or hamster IgG (Jackson ImmunoResearch, West Grove, PA). Fluorochrome-conjugated antibodies to mouse CD86 and CD40, and their corresponding isotype controls were purchased from BDPharMingen. One hundred thousand events per sample were collected from viable cells (as determined by light scatter profiles and PI staining) using a BD FACSAria flow cytometer, analyzed by FACSDiva (version 4.0) software (BD Biosciences, San Jose, CA) and histograms generated using FCS Express (version 3) software (De Novo Software, Thornhill, Ontario).

NFκB Assay

DC2.4 cells (1×10^6 cells per well) were stimulated with 1 µg/ml LPS and treated with 50 µg/ml notoginseng for 0, 30 and 90 minutes in 6-well plates. At each time point, cells were harvested and nuclear protein extracts prepared using the Active Motif nuclear lysis kit (Active Motif, Carlsbad, CA). Protein content of nuclear extracts was measured using a BCA protein assay kit (Pierce, Rockford, IL). NFκB p65 binding activity was determined using the Active Motif TransAM NFκB p65 kit according to the

manufacturer's directions. Briefly, 2.5 µg of nuclear protein was incubated for 1 hour in a 96-well plate coated with the NFκB consensus oligonucleotide sequence (5'-GGGACTTTCC-3'). NFκB p65 specific binding was quantified by absorbance (450nm) using a VersaMax spectrophotometer (Molecular Devices, Sunnyvale, CA).

Statistics

All statistical analyses were performed using GraphPad Prism 4.0a for the Macintosh (GraphPad Software, San Diego, CA). Differences between two means were analyzed by Student's t-test. Data sets with multiple comparisons were evaluated by one-way analysis of variance (ANOVA) with Dunnett's post test. Values of $p < 0.05$ were determined to be significant.

Results

Notoginseng inhibits LPS-induced TNF-α and IL-6 production by DC2.4 cells

In this study, the immunomodulatory effects of notoginseng were characterized using DC2.4 cells, a murine dendritic cell line. DC2.4 cells were stimulated with 1 µg/ml LPS and concomitantly treated with 0, 5, 25 or 50 µg/ml notoginseng. In unstimulated DC2.4 cells, notoginseng did not evoke either TNF-α or IL-6 release above basal levels even at the highest concentration used (Fig. 3-1A and 3-1B, respectively). The addition of LPS resulted in a 2-fold and 23-fold increase in the production of TNF-α and IL-6, respectively. Notoginseng significantly inhibited the production of both TNF-α and IL-6 in a concentration-dependent manner. At the highest concentration of notoginseng tested (50 µg/ml), LPS-induced TNF-α production was reduced approximately 2-fold. In

addition, nearly a 3-fold reduction in IL-6 production was observed at the 50 µg/ml concentration. Importantly, the reduction in LPS-induced cytokine production was not due to cytotoxicity, as viability of cells was similar to controls at all concentrations of notoginseng examined as assessed by trypan blue exclusion (data not shown).

The effects of notoginseng on TNF- α production are time-dependent

Notoginseng was evaluated for its potential to suppress TNF- α production by DC2.4 at varying time points relative to LPS stimulation. DC2.4 cells were either pretreated with 50 µg/ml of notoginseng for 24 hours prior to LPS addition (-24 hr timepoint), treated with notoginseng and LPS simultaneously (0 hr), or treated with notoginseng 8 hours after LPS addition (+8 hrs). All samples were collected 24 hours after LPS exposure. As expected, concomitant treatment of DC2.4 cell with LPS and notoginseng significantly suppressed the production of TNF- α after 24 hours of culture by more than 50 percent (Fig. 3-2). Pretreatment of DC2.4 cells with notoginseng for 24 hours resulted in a 20% decrease in TNF- α levels. In a separate experiment, removal of notoginseng from the medium after 24 hours of pre-treatment, but prior to LPS stimulation, also resulted in suppression of TNF- α following additional 24 hr incubation with LPS (data not shown). In contrast, addition of notoginseng 8 hours after LPS stimulation did not significantly inhibit TNF- α secretion, although a trend towards decreased levels was observed (Fig. 3-2).

Notoginseng modulates the production of TNF- α and IL-6 by DCs stimulated with additional TLR ligands

Dendritic cells can be activated to secrete pro-inflammatory cytokines by numerous pathogens expressing different TLR ligands. In this regard, the responsiveness of DCs to

Figure 3-1.

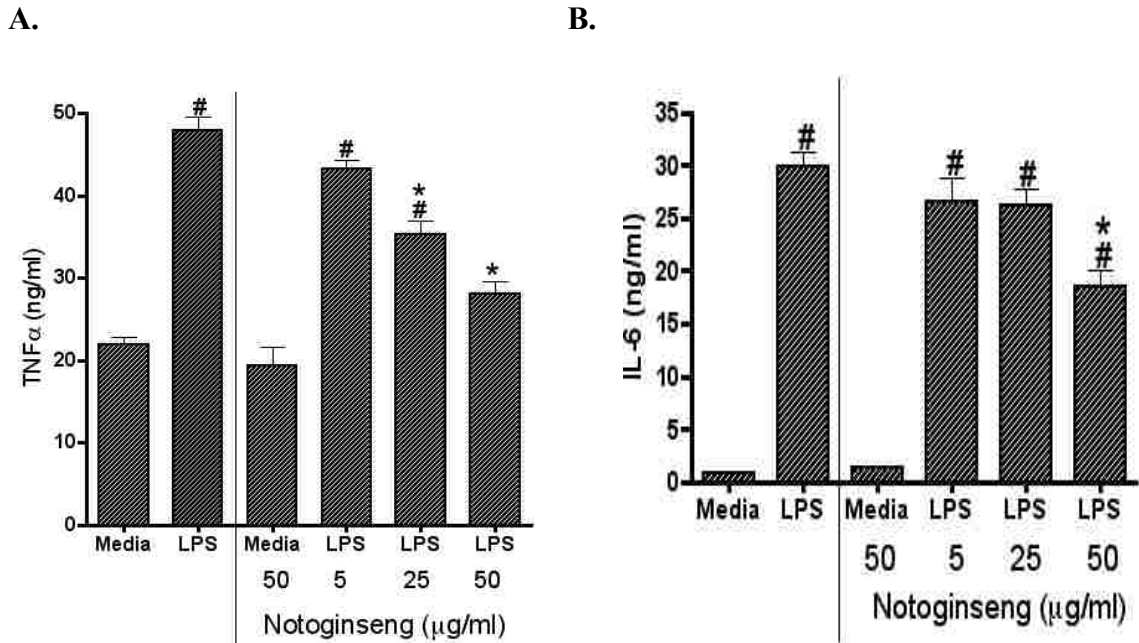


Figure 3-1. Notoginseng suppresses LPS-induced TNF- α and IL-6 production by DC2.4 cells. DCs were treated with 0, 5, 25 or 50 $\mu\text{g/ml}$ of notoginseng and/or LPS (1 $\mu\text{g/ml}$). Supernatants were collected after 24 hours and assayed for TNF- α (A) and IL-6 (B) production as described in the *Materials and Methods*. Data represents mean \pm SEM of three samples. Hash (#) indicates significant differences between LPS-stimulated and unstimulated cells; asterisk (*) indicates significant differences between the LPS-stimulated control- and notoginseng-treated samples ($p < 0.05$). Data are representative of three independent experiments.

Figure 3-2.

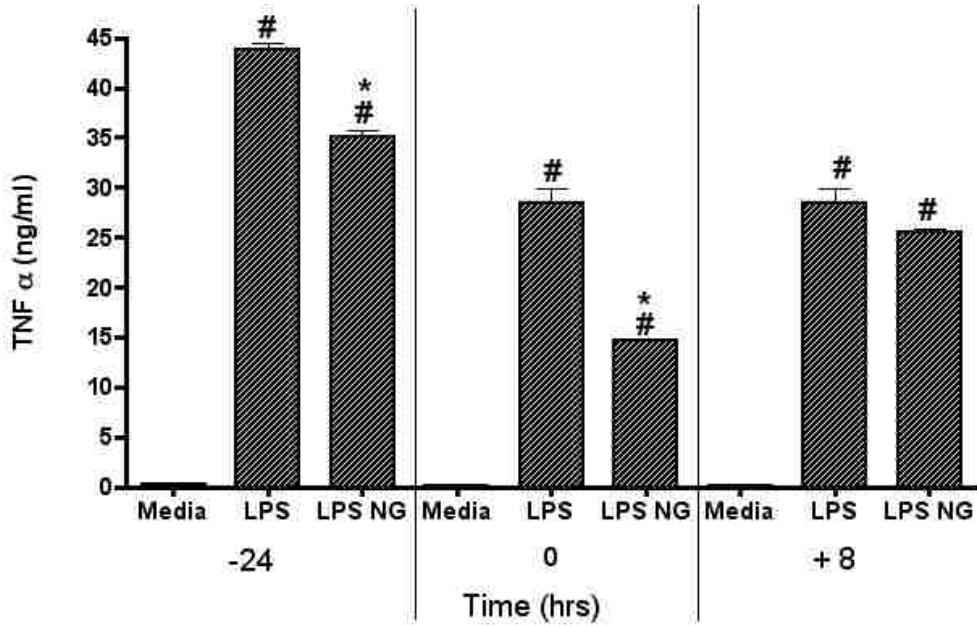


Figure 3-2. Variable notoginseng treatment affects LPS-induced production of TNF- α by DC2.4 cells. Cells were treated with 50 μ g/ml notoginseng (NG) at three different time-points: -24, 0 and 8 hours, relative to LPS stimulation. Supernatants were collected 24 hours after LPS addition and TNF- α levels measured by ELISA. Data represents mean \pm SEM of three samples. Hash (#) indicates significant differences between LPS-stimulated and unstimulated cells; asterisk (*) indicates significant differences between LPS-stimulated controls and LPS-stimulated, notoginseng-treated samples ($p < 0.05$). Data are representative of three separate experiments.

varied TLR ligands following exposure to notoginseng was examined. DC2.4 cells were stimulated with 1 µg/ml LPS, 0.5 µM CpG or 12.5 µg/ml poly(I:C) and concomitantly treated with 50 µg/ml notoginseng. The addition of LPS, CpG and poly(I:C) significantly increased the production of TNF-α by DC2.4 cells (Fig. 3-3A) (Sparwasser et al., 1998; Tsujimoto et al., 2006). Notoginseng inhibited the production of TNF-α by 46%, 36% and 50% for LPS, CpG and poly(I:C), respectively (Fig. 3-3A). There was a significant increase in the production of IL-6 in LPS and CpG stimulated samples while, barely detectable levels of IL-6 were present with poly(I:C) stimulation (Fig 3-3B). Notoginseng reduced the levels of IL-6 in the LPS- and CpG- stimulated samples (Fig 3-3B). In contrast, notoginseng did not inhibit the limited production of IL-6 by the poly(I:C)-stimulated DC2.4 cells (Fig 3-3B). As previously observed, the reduction in TLR-induced cytokine production was not due to cytotoxicity, as viability of notoginseng-treated cells was similar to controls as determined by trypan blue exclusion (data not shown).

Expression of TLR-induced costimulatory molecules by DCs is selectively affected by notoginseng

Because of the observed immunomodulatory effects of notoginseng on TLR-induced cytokine production, the potential for notoginseng to modulate costimulatory molecule expression on activated DCs was examined by flow cytometry. DC2.4 cells were treated concomitantly with each of the 3 TLR ligands and 50 µg/ml of notoginseng. The fluorescence intensity of two critical costimulatory molecules, CD40 and CD86, was assessed. Stimulation of DC2.4 cells with LPS, CpG and poly (I:C) significantly increased the expression of CD40 (Table 3-1 and Fig. 3-4) (Sparwasser et al., 1998) . CD86 expression was significantly increased following LPS and poly(I:C) stimulation,

Figure 3-3.

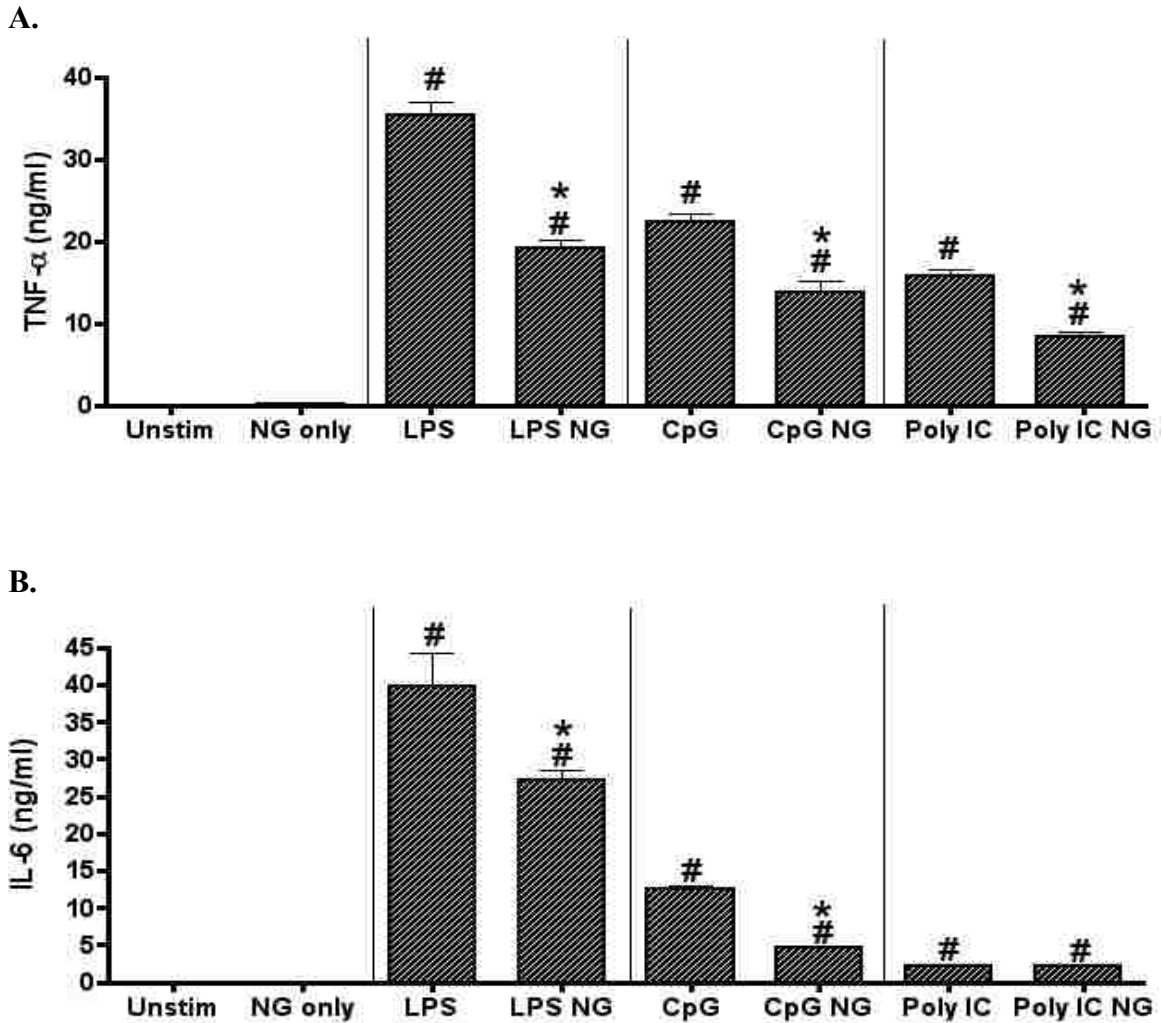


Figure 3-3. Notoginseng selectively reduces TLR ligand-induced production of TNF- α and IL-6 by DC2.4 cells. DCs were unstimulated or stimulated with LPS, CpG or poly(I:C) and concurrently treated with 50 μ g/ml notoginseng (NG) for 24 hours. Supernatants were collected and assayed for TNF- α and IL-6 production by ELISA. Data represents mean \pm SEM of three samples. Hash (#) indicates significant differences between stimulated and unstimulated cells; asterisk (*) indicates significant differences between the TLR ligand-stimulated control- and notoginseng-treated samples ($p < 0.05$). Data are representative of three independent experiments.

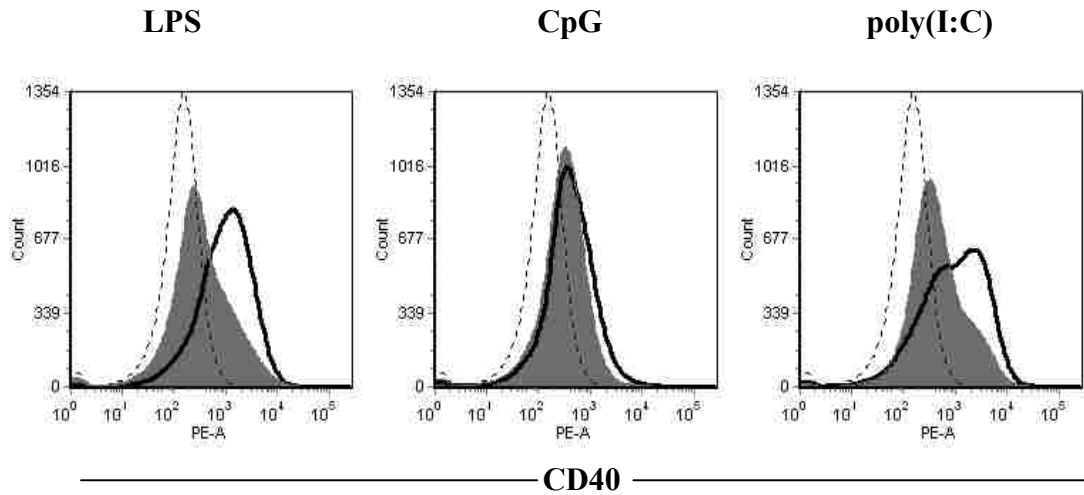
Table 3-1 Notoginseng selectively alters TLR-induced costimulatory molecule expression on DC2.4 cells.

	CD40^a	CD86
Unstimulated	192 ± 2	1224 ± 28
LPS	1484 ± 61[#]	1630 ± 52[#]
LPS + Notoginseng	932 ± 35^{#*}	1295 ± 115^{#*}
CpG	611 ± 34[#]	1141 ± 33
CpG + Notoginseng	512 ± 13^{#*}	1408 ± 28^{#*}
Poly(I:C)	1627 ± 83[#]	1366 ± 49[#]
Poly(I:C) + Notoginseng	1098 ± 40^{#*}	929 ± 2^{#*}

DC2.4 cells were not stimulated or stimulated with LPS (1µg/ml), CpG (0.5µM) or poly(I:C) (12.5 µg/ml) and concomitantly treated with notoginseng (50 µg/ml) for 24 hours. ^aMean Channel Fluorescence (MCF) was determined by flow cytometry. Data shown are representative of three independent experiments. Error bars indicate mean ± SEM of five samples; #, *p*<0.05 for the comparison of unstimulated and TLR-activated cells; *, *p*<0.05 for the comparison of TLR-stimulated cells treated with notoginseng or untreated.

Figure 3-4.

A.



B.

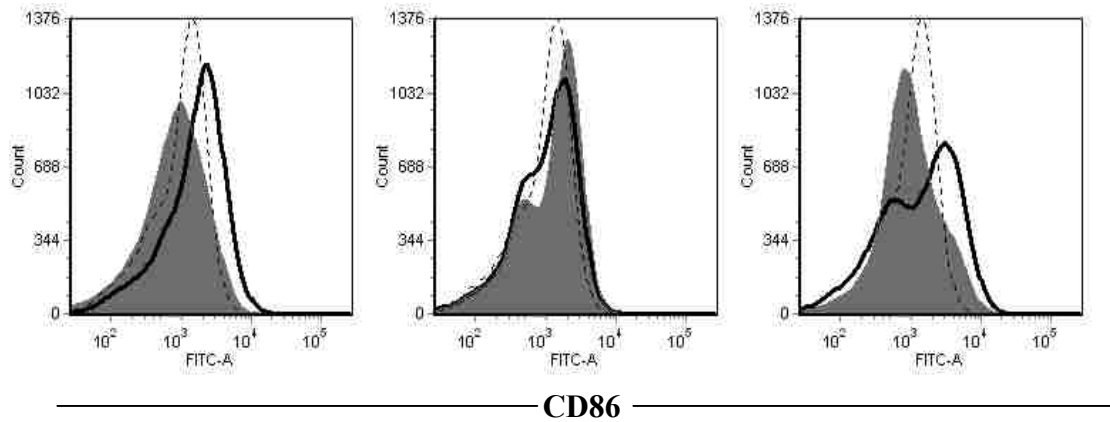


Figure 3-4. Notoginseng inhibits the expression of costimulatory molecules on DC2.4 cells following stimulation with TLR ligands. Cells were unstimulated (dashed line) or stimulated (black line) with LPS, CpG or poly(I:C) and concomitantly treated with 50 µg/ml notoginseng (gray histogram) for 24 hours. Representative histograms demonstrating the cell surface expression of CD40 (A) and CD86 (B) on activated DC2.4 cells were determined by FACS analysis. Data are representative of three independent experiments, each consisting of a minimum of three samples per treatment group.

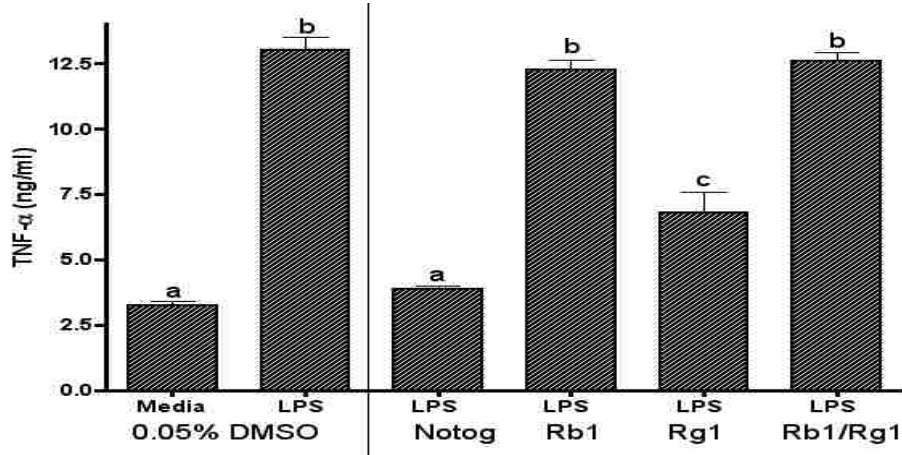
but not with CpG stimulation (Fig. 3-4). Treatment of unstimulated DC2.4 cells with notoginseng did not affect CD40 expression; however, CD86 levels were decreased following exposure to the herbal extract alone (data not shown). Notoginseng treatment decreased CD40 expression on DCs stimulated with all examined TLR ligands (Fig. 3-4). Likewise, CD86 expression was reduced on notoginseng-treated DC2.4 cells that were stimulated by LPS or poly(I:C). In contrast, there was an increase in CD86 expression in CpG-stimulated, notoginseng-treated samples.

The effects of purified ginsenosides Rb1 and Rg1 on TNF- α and IL-6 production in LPS-stimulated DC2.4 cells

Ginsenosides are believed to be responsible for the biological activity of notoginseng. The notoginseng extract used in our studies is comprised of several ginsenosides, primarily Rb1 (35%) and Rg1 (34%). Therefore, the potential inhibitory effects of the purified ginsenosides Rb1 and Rg1 on LPS-induced TNF- α and IL-6 production were evaluated in DC2.4 cells. To more closely mimic the approximate concentrations of ginsenosides present in the whole extract, Rb1 and Rg1 were standardized to concentrations found in 50 $\mu\text{g/ml}$ of the whole notoginseng extract. Following LPS activation of DC2.4 cells, Rg1 significantly inhibited both TNF- α and IL-6 production (Fig. 3-5A and 3-5B). In contrast, Rb1 only inhibited the production of IL-6 and was less potent when compared to Rg1. Neither ginsenoside reduced the production of either TNF- α or IL-6 as effectively as the whole notoginseng extract. Interestingly, combination of the purified Rb1 and Rg1 compounds did not significantly decrease the production of either cytokine.

Figure 3-5.

A.



B.

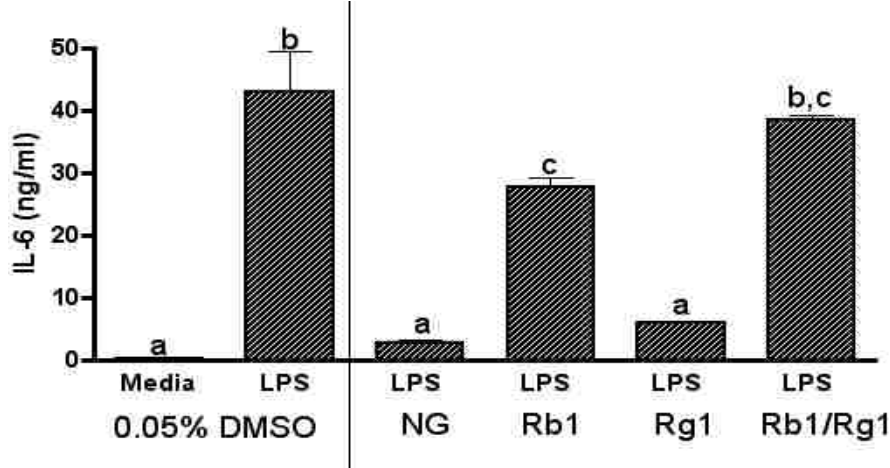


Figure 3-5. The production of TNF- α and IL-6 by DC2.4 cells is selectively suppressed by the whole notoginseng extract and purified ginsenosides. LPS-stimulated cells were treated with 50 μ g/ml whole notoginseng extract (NG) or purified Rb1 and/or Rg1 ginsenosides. Supernatants were collected after 24 hours and assayed for TNF- α (A) and IL-6 (B) production by ELISA. Data represents mean \pm SEM of 3 samples. Statistically significant differences ($p < 0.05$) between treatment groups are indicated by different letters while groups sharing letters are not significantly different. Data are representative of two separate experiments.

Notoginseng does not alter LPS-induced NFκB p65 activity in DC2.4 cells

Translocation of NFκB to the nucleus is important for TLR ligand-induced inflammatory mediator production by dendritic cells. Given that notoginseng decreased the activation of DCs in our previous experiments and ginseng extracts have been shown to modulate NFκB activation in other immune cells, the effects of notoginseng on NFκB p65 nuclear levels were examined. DC 2.4 cells were either unstimulated (0 min), or stimulated with LPS for 30 or 90 mins and concurrently treated with 50 µg/ml notoginseng. Samples were then harvested and the nuclear fraction evaluated for NFκB p65 binding activity. LPS stimulation of DC2.4 cells resulted in increased NFκB p65 levels at both timepoints (data not shown). However, notoginseng did not significantly affect NFκB p65 activity in the cultured DCs.

Discussion and conclusion

Dendritic cells play an important role in the innate immune response to microbial pathogens (Granucci et al., 2005). Several studies have reported the effects of ginseng on various immune cell types; however, no information exists describing its effects on murine DCs. Previous studies on the effects of notoginseng on innate immunity have primarily focused on LPS-activated leukocytes. For this reason, we chose to examine the potential immunomodulatory effects of notoginseng on murine dendritic cells following stimulation with several TLR ligands, including LPS. Specifically, the effects of notoginseng on DC2.4 cells were examined following activation of TLR3, TLR4 and TLR9 by poly (I:C), LPS and CpG, respectively. The production of two critical inflammatory cytokines TNF-α and IL-6 were evaluated, as they are produced early

during DC activation. Moreover, TNF- α is involved in the maturation of DCs. Along with IL-6, TNF- α can rapidly induce expression of costimulatory molecules such as CD86 on DCs thereby enhancing their interactions with T cells (Fujii et al., 2004). As LPS is a prototypical inflammatory stimulus, the effects of notoginseng were initially characterized following TLR4 activation. Notoginseng decreased the production of TNF- α and IL-6 by LPS-activated DC2.4 cells. Furthermore, these effects were demonstrated to be time- and concentration-dependent. Both pretreatment and concomitant notoginseng treatment significantly reduced TNF- α production. The effects were evident even when notoginseng was removed from the media prior to LPS stimulation of the dendritic cells (data not shown). These results suggest that notoginseng may bind to an external receptor on the DC2.4 cells, may be taken up by the DCs, or potentially both. Additionally, the actions of notoginseng most likely occur via intracellular changes, and not by binding LPS and preventing it from activating its receptor, as its presence is not required for the inhibition of TNF- α . When DCs were exposed to notoginseng 8 hours after LPS stimulation, no significant inhibitory effects were identified on TNF- α production. These results suggest that notoginseng may affect early signaling events in DCs following LPS-induced activation.

As previously mentioned, activation of DC can occur by a variety of pathogens through different TLRs (Napolitani et al., 2005). In these experiments, LPS, CpG and Poly (I:C) were used to model stimulation of DCs by both bacterial and viral pathogens (Sioud, 2006). Notoginseng reduced the production of TNF- α by DC2.4 cells in response to each of the TLR ligands tested while IL-6 production was reduced following LPS and CpG, but not poly(I:C) stimulation. Because the TLR-induced production of both of these

pro-inflammatory cytokines was not completely abolished by notoginseng, DC function may not be significantly diminished. Thus, notoginseng may inhibit the inflammatory responsiveness of DCs without significantly affecting their ability to initiate adaptive immunity. If so, this medicinal herb might be expected to benefit individuals infected with microbial pathogens by limiting the production of inflammatory mediators such as TNF- α and IL-6 without affecting the generation of pathogen-specific adaptive immunity. This possibility is consistent with a previous report demonstrating that notoginseng-treated mice were less susceptible to the ill effects of experimental sepsis, effects which the authors attributed to a decreased inflammatory response to infection (Ahn et al., 2006).

The expression of TLRs can vary within different DC subpopulations (Krug et al., 2001). Currently, no information exists on the levels of expression for different TLRs in DC2.4 cells. Further experiments are therefore needed to characterize the level of expression of TLR3, TLR4 and TLR9 in DC2.4 cells to permit better understanding of how notoginseng may affect their expression and function. Also, it is becoming widely accepted that pathogens will trigger multiple TLRs during the course of infection. Thus, additional experiments designed to evaluate the effects of notoginseng on DCs stimulated with multiple TLR ligands would be expected to greatly enhance our understanding of the innate immune responsiveness of DCs.

Interactions between accessory/costimulatory molecules on DCs and their ligands expressed on T cells are critical for the full activation of T cells (Banchereau et al., 2000a). Ligation of CD40 on DCs acts as a maturation signal, enhancing antigen presentation and the expression of other co-stimulatory molecules; while CD86 is

believed to be the most critical molecule for the amplification of T cell responses (Banchereau et al., 2000a; Fujii et al., 2004). Previous studies from our laboratory demonstrated that notoginseng reduced the LPS-induced expression of both CD40 and CD86 on RAW 264.7 cells (Rhule et al., 2006b). Similarly, CD86 and CD40 expression on LPS-activated DC2.4 cells was reduced using similar concentrations of notoginseng. Both molecules on DCs were also reduced by notoginseng treatment following poly(I:C) stimulation. In contrast, CD40 but not CD86 expression was reduced on DC 2.4 cells stimulated with CpG. Current studies in our laboratory are evaluating the functional significance of the notoginseng-induced changes in costimulatory molecule expression on DCs.

Ginsenosides are unique to the ginseng species and are believed to be the biologically active components of notoginseng. Because of the wide variability in the types and concentrations of ginsenosides present in ginseng extracts, it is crucial to define the immunomodulatory potential of different ginsenosides. In this study, the ginsenoside Rg1 inhibited the production of both TNF- α and IL-6, while Rb1 only affected the production of IL-6. Unexpectedly, when Rg1 was combined with Rb1, the inhibitory effect of Rg1 was lost. This result could be due to a number of factors including differential effects of Rb1 and Rg1 on cell membrane permeability, on the activation of disparate receptors and/or signal transduction pathways, or perhaps via the partial antagonism of Rg1 by Rb1 (Attele et al., 1999). These possibilities are consistent with a number of studies reporting that complex ginseng extracts can differentially affect immune cell function based on their specific ginsenoside profiles (Cho et al., 2002a; Cho

et al., 2001; Guermonprez et al., 2002; Joo et al., 2005). Future studies will therefore be required to elucidate the complex nature of ginsenoside interactions in dendritic cells.

The transcription factor NF κ B plays an important role in the regulation of multiple signaling pathways that control the activation of many immune cells (Celec, 2004a; Glass and Ogawa, 2006a). TLR ligands activate NF κ B proteins in DCs and subsequently affect their fate and function (Wang et al., 2007). Several studies with ginseng suggest that it may inhibit NF κ B activation (Ahn et al., 2006; Chung et al., 1998; Keum et al., 2003b). In our studies, notoginseng did not affect NF κ B p65 activation. Thus, it is likely that this herbal exerts its anti-inflammatory effects through other pathways such as MAPK and AP1, or through other NF κ B family members such as RelB or cRel. Alternatively, because DC2.4 cells are an immortalized cell line that are not terminally differentiated, they may respond differently than primary DCs following exposure to notoginseng. This observation is consistent with recent studies in our laboratory in which notoginseng reduced NF κ B p65 nuclear levels and activity in LPS-stimulated bone marrow-derived dendritic cells (manuscript in preparation).

In summary, this study demonstrates that notoginseng inhibits the production of TNF- α and selectively decreases IL-6 production by DCs following TLR activation. Similarly, notoginseng differentially affected the expression of the costimulatory molecules CD40 and CD86 on DCs following activation by different TLR ligands. Collectively, our results demonstrate that notoginseng can decrease the inflammatory responsiveness of DCs to bacterial or viral stimuli. Further studies are needed to examine directly if the notoginseng-induced decreases in cytokines and accessory molecules by DCs alters their ability to initiate T cell-dependent adaptive immunity.

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An ethanolic extract of the medicinal herb, *Panax notoginseng*, inhibits innate but not adaptive immune function of murine dendritic cells.

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Abstract

Dendritic cells (DCs) play an important role in facilitating the interaction between the innate and adaptive arms of the immune system. Many strategies have been used to manipulate DCs for therapeutic purposes. The medicinal herbal *Panax notoginseng* has been extensively studied in macrophages and other cell types for its innate immune effects. However, limited information exists on the effects of notoginseng on dendritic cells, and their ability to control the adaptive immune response. In this regard, the immunomodulatory effects of notoginseng were investigated using murine bone marrow-derived dendritic cells (BMDCs). BMDCs were stimulated with 1 µg/ml of LPS and either pre-treated or concurrently treated with 0-200 µg/ml notoginseng. LPS-induced production of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and IL-12 was decreased by notoginseng. The cell surface expression of CD40 was reduced in cultures concurrently stimulated with LPS and treated with notoginseng, while CD86 and MHC II levels were decreased only following pre-treatment of BMDCs with notoginseng prior to LPS stimulation. Phagocytosis of FITC-ovalbumin was also reduced in notoginseng-treated BMDCs. Functionally, T cell proliferation in response to notoginseng-treated, antigen (Ag)-loaded BMDCs was not significantly altered *in vitro* or *in vivo*. Mechanistically, notoginseng decreased BMDC activation independent of glucocorticoid receptor GCR activation; however, reduced nuclear levels of NFκB p65 were observed in notoginseng-treated BMDCs when compared to controls. Collectively, notoginseng reduced the production of inflammatory mediators by BMDCs without altering their ability to interact with CD4⁺ T cell in an antigen-specific manner. Therefore, our studies demonstrate that notoginseng alters the innate but not adaptive functions of DCs.

Introduction

The induction of T cell-mediated immunity depends on the successful interaction of Ag-bearing antigen presenting cells (APCs) with Ag-specific T cells (Di Nicola and Lemoli, 2000). DCs are recognized as the primary APCs of the immune system. These cells also provide an integral link between innate and adaptive immunity (Granucci et al., 2003). They are important in the mediation of inflammation and have been implicated in numerous inflammatory diseases (Kuipers and Lambrecht, 2004; Sharma and Li, 2006b; Thomas et al., 1999). Activated DCs produce various cytokines such as TNF- α , IL-1 β , IL-6 and IL-12, which affect both microbial pathogens and leukocytes. These cytokines are also influential in the generation of several inflammatory conditions including atherosclerosis and rheumatoid arthritis (Andreakos et al., 2004a; Munz et al., 2005). Additional responses of DCs following activation include upregulation of adhesion and costimulatory molecules such as CD40 and CD86, and also major histocompatibility complexes (MHC class I and II) on their cell surface (McLellan et al., 2000). These molecules are essential for productive Ag-specific interactions to occur between DCs and T cells (Fujji et al., 2004).

Many DC responses are regulated at the transcriptional level and, in particular, by the transcription factor, nuclear factor kappa B (NF κ B) (West et al., 2004). When activated by classic inflammatory stimuli such as TNF- α and LPS, NF κ B is released from the inhibitory protein I κ B and translocates from the cytoplasm to the nucleus where it binds and induces transcription of specific target genes. This results in the upregulation of numerous immune and inflammatory genes. Pro-inflammatory mediator induction by NF κ B activation can be affected by the glucocorticoid receptor (GCR) (Glass and

Ogawa, 2006b). Signaling through the GCR results in potent inhibition of the inflammatory response as well as attenuated DC function (Glass and Ogawa, 2006b; Valledor and Ricote, 2004).

Several natural and synthetic chemicals are capable of modulating the immune system (Patwardhan and Gautam, 2005). Currently, most compounds used to combat immunologic diseases are synthetic and biosynthetic pharmaceutical products. However, the use of herbal products is increasing, with consumer sales reaching previously unseen numbers (Paterson and Anderson, 2005). In spite of this increased usage, the effects of many of these herbals on the immune system have not been well characterized. As many of these herbals have the potential to modulate the immune system, it is important to understand their mechanisms of action and potential immunotoxic effects.

Ginseng has been used as a medicinal for over 2000 years in Asian countries and is now becoming increasingly popular in Western countries such as the United States (Sato and Miyata, 2000). Numerous studies have shown that ginseng possesses immunomodulatory properties (Hofseth and Wargovich, 2007; Jie et al., 1984; Liou et al., 2005). These biological effects are most likely mediated by ginsenosides, the purported therapeutic phytochemicals in ginseng (Dong et al., 2003a). There are thirteen known species of ginseng with the *Panax ginseng*, *Panax quinquefolium* and *Panax notoginseng* being the three most popular medicinal species. Of these three, *Panax notoginseng* possesses the highest concentration of ginsenosides (Harkey et al., 2001b). Several studies exist describing the effects of *Panax notoginseng* and ginseng derivatives on various immune cell types. These studies primarily involve the anti-inflammatory effects of notoginseng, specifically its reduction of cytokine production and inflammatory

enzymes such as iNOS and COX-2 (Park et al., 2005). Currently, no information exists regarding the effects of notoginseng on the innate function of murine DCs or their capacity to interact with T cells in an Ag-specific manner.

To address this deficiency, we have examined the immunomodulatory effects of *Panax notoginseng* in murine bone marrow-derived dendritic cells (BMDCs). BMDCs were stimulated with LPS and concurrently treated with notoginseng. Production of the pro-inflammatory cytokines TNF- α , IL-1 β , IL-6 and IL-12 and expression of the cell surface molecules CD40, CD86 and MHCII were analyzed following notoginseng exposure. The effects of notoginseng on antigen uptake and Ag-specific activation of T cells were also evaluated. Studies have suggested that alteration of NF κ B activation in ginseng-treated macrophages may be responsible for decreased production of inflammatory mediators (Oh et al., 2004a; Park et al., 2005). Therefore, the effects of notoginseng on NF κ B activation were investigated in BMDCs. Additionally, the GCR blocker RU486 (mifepristone) was utilized to determine whether notoginseng's activity occurred through GCR-mediated mechanisms. Our results demonstrate that notoginseng inhibits the innate immune responsiveness of DCs without altering their antigen-specific adaptive immune function.

Materials and methods

Mice

C57BL/6 mice aged 6-12 weeks old were bred and maintained in the animal research facilities at the University of Montana. Mice were housed under specific pathogen-free conditions and maintained on 12-hour dark/light cycles. Standard laboratory food and

water were provided ad libitum. All protocols for the use of animals were approved by the University of Montana Institutional Animal Care and Use Committee.

BMDCs

Bone marrow cells (BMCs) were collected from the femur and tibia of C57BL/6 mice for *in vitro* use, by flushing out the bone marrow with cRPMI using a 30-gauge needle. BMCs were then layered on a density gradient using lympholyte reagent (Cedarlane laboratories limited, Ontario, Canada) and centrifuged at 800 X g for 20 minutes. The lymphocyte layer was collected and washed with cRPMI. To generate myeloid-derived BMDCs, BMCs were grown in cRPMI with 30 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF) (Leinco, St Louis, Missouri) at 1×10^6 cells per ml in T75 flasks. Media and growth factor were removed and replenished on days 3 and 5. Cells were harvested after 7 days and DCs purified using (90% CD11c⁺) CD11c-APC antibodies and anti-APC Miltenyi magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions.

Chemicals

Noto-GTM extracts from the plant *Panax notoginseng* (Burk.) F.H. Chen ex C.Y. Wu & K.M. Feng were kindly supplied by Technical Sourcing International, Inc. (TSI, Missoula, MT). Notoginseng was extracted from the root of the plant using ethanol and standardized to contain Rb1 and Rg1 ginsenosides at 35 and 34% of the whole extract, respectively. Quantification of Rb1 and Rg1 in the notoginseng extract was determined by high-performance liquid chromatography analysis by TSI. Certification of analyses was approved by Xia Ronglong (QA manager, TSI). *Escherichia coli* (E. coli) and

Salmonella enterica were undetectable in the notoginseng preparation (unpublished data). The extract was dissolved in complete media (see below) and subsequently sterile-filtered through a 0.22 μ M Millipore membrane. Lipopolysaccharide (LPS) from *E. coli* (055:B5); whole ovalbumin, RU486 and dexamethasone (DEX) were obtained from Sigma-Aldrich. Custom synthesized OVA₃₂₃₋₃₃₉ peptide was purchased from Mimotopes, San Diego, CA.

Cell Activation and Treatment

BMDCs (1×10^6 cells per well) were stimulated with 1 μ g/ml LPS and treated with 0, 100, 150 or 200 μ g/ml notoginseng for 24 hrs at 37 °C and 5% CO₂ in 6-well plates. After 24 hrs, cells were harvested for RT-PCR and FACS analyses and supernatants were collected for evaluation by ELISA. BMDC treatment with the GCR antagonist, RU486, and the GCR agonist, DEX, as described in previously published conditions (Pan et al., 2001). Briefly, RU486 (1 μ M) was added to BMDCs 15 minutes prior to notoginseng or DEX (100 nM) treatment and stimulation with LPS. DMSO (less than 0.1%) was added to unstimulated and LPS-stimulated samples to serve as controls in these experiments, as DEX and RU486 were dissolved in DMSO. For BMDC antigen processing and presentation experiments, notoginseng was removed from the media after 24 hrs and replaced with fresh media. Cells were then pulsed with 100 μ g/ml whole OVA or 50 μ g/ml OVA₃₂₃₋₃₃₉ peptide for 24 hrs. BMDCs were subsequently harvested, counted and prepared for assay. The viability of BMDCs used in all experiments was greater than 90% as determined by Trypan blue exclusion.

Cytokine Assays

Levels of TNF- α , IL-1 β , IL-2, IL-6 and IL-12 in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA). Samples were analyzed per the manufacturer's instructions using mouse cytokine-specific BD OptEIA ELISA kits (BD PharMingen, San Diego, CA).

Flow Cytometry

Detection of accessory molecule expression on BMDCs by fluorescent activated cell sorting (FACS) analysis was performed as previously described (Shepherd et al., 2001). Briefly, BMDCs were harvested and washed with PAB (1% bovine serum albumin and 0.1% sodium azide in PBS). Cells were blocked with 30 μ g of purified rat and/or hamster IgG (Jackson ImmunoResearch, West Grove, PA) for 10 minutes to eliminate non-specific staining. Optimal concentrations of fluochrome-conjugated monoclonal antibodies were used to stain cells for an additional 10 minutes. The antibodies used in these experiments were CD86-APC, CD40-PE, MHCII-PE, CD4-PE, CD11c-APC, CD44-PB, CD62L-PE and their corresponding isotype controls (BDPharMingen, San Diego, CA). One to five hundred thousand viable cells per treatment (as determined by light scatter profiles and propidium iodide staining) were analyzed using a BD FACSAria flow cytometer and FACSDiva software (BD Biosciences, San Jose, CA).

NF κ B Activity Assay

BMDCs (1×10^6 cells per well) were stimulated with 1 μ g/ml LPS and treated with 150 μ g/ml notoginseng for 0, 1 or 2 hrs at 37 $^{\circ}$ C and 5% CO $_2$ in 6-well plates. At each time-

point, cells were harvested, lysed and proteins extracted using the ActiveMotif nuclear lysis kit (Active Motif, Carlsbad, CA). The protein content of the nuclear extract was measured using a BCA protein assay kit (Pierce, Rockford, IL). Nuclear protein (2.5 µg/ml per well) was then added to the Active Motif TransAM NFκB p65 kit (Active Motif, Carlsbad, CA) and assayed according to the manufacturer's specifications. Briefly, 2.5 µg of nuclear protein was incubated in a 96-well plate containing the NFκB consensus site (5'-GGGACTTTCC-3') for 1 hour. This was followed by a 1-hour incubation with a primary antibody that recognizes NFκB p65 bound to target DNA. The peroxidase-conjugated secondary antibody was added for an hour, after which samples were developed and analyzed by spectrophotometry.

Immunoblotting

In some experiments, nuclear extracts from the treated BMDCs were also assayed for NFκB p65 protein by Western blotting. For these assays, 10 µg of protein was loaded onto a SDS-PAGE gel and blotted onto 0.2 µm polyvinylidene difluoride transfer membrane (Biorad, Hercules, CA). Membranes were blocked using 10% nonfat dried milk in PBS with 0.05% Tween (PBST), then incubated overnight at 4⁰C with either antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) to NFκB p65 antibody (1:500) or β-actin (1:4000). The membrane was washed and incubated with either peroxidase-conjugated anti-rabbit IgG (Jackson ImmunoResearch, 1:1000) or anti-mouse IgG (Southern Biotechnologies). Membranes were exposed for 1-5 mins and processed using Fuji film imaging software.

In vitro Antigen Presentation Assay

BMDCs were untreated or pretreated with notoginseng for 24 hours, washed to remove notoginseng, then loaded with Ag (whole ovalbumin at 100 µg/ml or ova peptide at 50 µg/ml) overnight. T cells were isolated from the spleens of OT-II T cell receptor transgenic mice and purified using CD4-PE antibodies (BD Pharmingen, San Diego, CA) and anti-PE beads (Miltenyi Biotec, San Diego, CA). T cells (2×10^5) were co-cultured with BMDCs (2×10^5) cells in 96 well plates. After 72 hours, cells were pulsed with 1µCi [3 H] thymidine (Amersham, Piscataway, NJ) for 24 hours. Cells were harvested and [3 H] thymidine incorporation measured via liquid scintillation.

Adoptive Transfer of Ag-loaded donor DCs and Ag-specific T cells

The protocol for adoptive transfer was modified from a previously described method (Shepherd et al., 2000). Briefly, BMDCs were exposed to 100 µg/ml of notoginseng for 24 hours. Cells were treated with 50 µg/ml of OVA peptide for an additional 24 hours. Splenic OTII CD4⁺ T cells were harvested from OT II/Thy1.1 mice and enriched using Miltenyi magnetic beads. OT II T cells (2×10^6 per mouse) were resuspended in HBSS and injected iv into age- and sex-matched, CD45.1 congenic host mice on day -1 relative to immunization. Host-mice were immunized on day 0 with ova peptide-loaded BMDCs (1×10^6) via rear footpad injection. On day 4 post-immunization, popliteal lymph node cells were harvested from the host mice and analyzed by flow cytometry. Brachial lymph node cells were also harvested and evaluated as unactivated controls. Subsequent analysis of the Ag-loaded BMDCs and Ag-specific T cells was based on the phenotypic expression of CD45.2⁺/CD11c⁺ donor dendritic cells and CD4⁺/Thy1.1⁺ OT II T cells,

respectively. Cells from the host were excluded based on their expression of CD45.1 and Thy 1.2 cell surface proteins.

Statistics

All statistical analyses were performed using GraphPad Prism 4.0a for the Macintosh (GraphPad Software, San Diego, CA). Data represents the mean \pm SEM of 3 samples for 3 independent experiments unless otherwise stated. Differences between treatment groups were analyzed by Student's t-test. Data sets with multiple comparisons were evaluated by one-way analysis of variance (ANOVA) with Dunnett's test. Values of $p < 0.05$ were determined to be significant.

Results

Notoginseng suppresses the LPS-induced production of pro-inflammatory cytokines by BMDCs

Cytokines are important mediators in the orchestration of an immune response. To investigate the effects of notoginseng on the production of cytokines by dendritic cells, BMDC were stimulated with 1 $\mu\text{g/ml}$ LPS and concurrently treated with notoginseng. As shown in Fig. 4-1, activation of BMDCs with LPS resulted in significant increases in the production of TNF- α , IL-1 β , IL-6 and IL-12, when compared to unstimulated controls. At the highest concentration of notoginseng tested (200 $\mu\text{g/ml}$), the LPS-induced production of IL-1 β , IL-6 and IL-12 was decreased by approximately 90%. Notoginseng also reduced TNF- α production by BMDCs although to a lesser degree than observed for the other pro-inflammatory cytokines. No cytotoxicity was observed at any of the concentrations of notoginseng used in our study as assessed by Trypan blue exclusion

Figure 4-1.

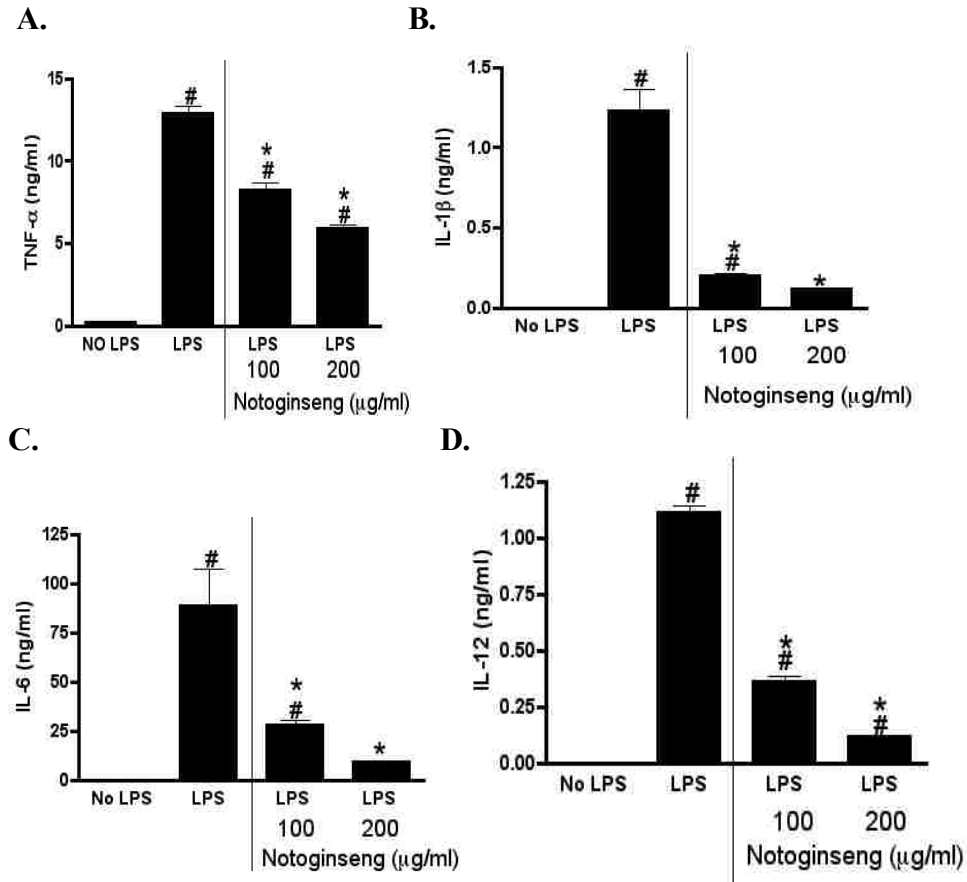


Figure 4-1. Notoginseng modulates LPS-induced cytokine production by BMDC. Cells were stimulated with LPS (1 μ g/ml) and concomitantly treated with 0, 100 or 200 μ g/ml of notoginseng. Supernatants were collected after 24 hours and assayed for TNF- α , IL-1 β , IL-6 and IL-12 levels by ELISA. Data represent mean \pm SEM of 3 samples. # Indicates significant differences between stimulated and unstimulated cells ($p < 0.05$), * indicates significant differences between the LPS-stimulated, control- and notoginseng-treated samples ($p < 0.05$). Data are representative of 3 independent experiments.

(data not shown). Also, notoginseng did not significantly affect cell recoveries of BMDCs when compared to control treated samples.

BMDC expression of key accessory molecules is affected by notoginseng

To determine the effect of notoginseng on accessory molecule expression on DCs, BMDCs were stimulated with LPS and concurrently treated with 200 µg/ml of notoginseng for 24 hours. Activation markers on BMDCs were analyzed by flow cytometry. A significant increase in CD40, CD86 and MHC II cell surface expression was observed following LPS stimulation of BMDCs (Fig. 4-2 and Table 4-1). CD40 expression was reduced by 58% on BMDCs treated concurrently with notoginseng and LPS while CD86 and MHC II were unaffected by the herbal treatment. In some experiments, cells were pretreated with notoginseng for 20 hrs prior to LPS stimulation to determine if notoginseng exposure prior to activation differentially affected cell surface molecule expression. Pretreatment of BMDCs resulted in an even greater reduction in the expression of CD40, while also significantly decreasing CD86 and MHCII expression.

Reduced uptake of FITC-conjugated whole ovalbumin by notoginseng-treated BMDCs

To assess whether notoginseng reduced antigen uptake, a primary function of dendritic cells, BMDCs were incubated with FITC-conjugated whole ovalbumin (FITC-ova). Fluorescence intensity was used to assess the phagocytosis of FITC-ova by BMDCs via both flow cytometry and fluorescence microscopy analyses (Fig. 4-3). A decrease in fluorescence intensity was observed in both the 50 and 100 µg/ml notoginseng-treated groups when compared to controls as determined by light microscopy (Fig. 4-3A). This effect was quantitatively confirmed by flow cytometry, as a 3-fold reduction in mean

Table 4-1. Notoginseng differentially affects the expression of LPS-induced accessory molecules on BMDCs.

	CD40 MCF^b	CD86 MCF	Percent MHC II^{+c}
Unstimulated^a	683 ± 12	5991 ± 105	17 ± 1.7
LPS	8985 ± 1305 ^{#d}	11280 ± 488 [#]	27 ± 0.8 [#]
LPS + NG	3750 ± 172 ^{#*}	10210 ± 386 [#]	24 ± 1.0 [#]
LPS + NG PRE-TREAT	2266 ± 105 ^{#*}	8872 ± 384 ^{#*}	11 ± 0.6 ^{#*}

^a BMDC were either unstimulated, stimulated with LPS only, stimulated with LPS and concomitantly treated with notoginseng (200 µg/ml), or pre-treated with notoginseng for 20 hours prior to LPS stimulation. Cells were harvested 24 hour following stimulation with LPS.

^b Mean Channel Fluorescence (MCF) as determined by flow cytometry

^c Percentage of BMDCs expressing high levels of MHC II as determined by flow cytometry.

^d Data shown are representative of three independent experiments. Error bars indicate mean ± SEM of 3 samples; #, $p < 0.05$ for the comparison of unstimulated and LPS-activated cells; *, $p < 0.05$ for the comparison of LPS-stimulated cells to notoginseng-treated, LP-stimulated cells.

Figure 4-2.

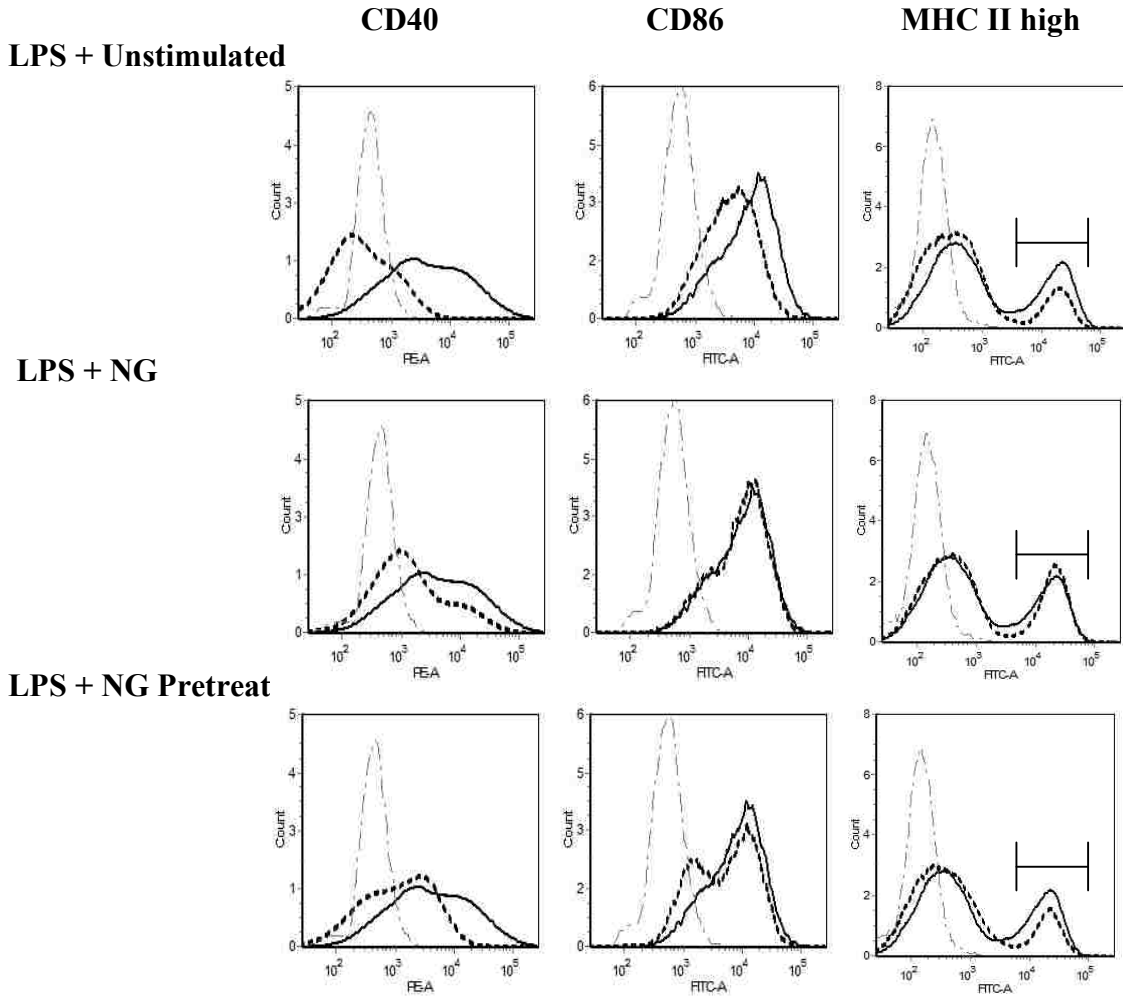


Figure 4-2. Effects of notoginseng on LPS-induced expression of accessory molecules on BMDCs. Cells were treated with notoginseng (200 $\mu\text{g}/\text{ml}$) either concurrently or 20 hours prior to LPS stimulation. Cells were stimulated with LPS for 24 hours and CD40, CD86 and MHC II expression was assessed by flow cytometry. The broken lines represent unstained controls; solid lines represent LPS-stimulated samples, and dotted lines represent LPS-stimulated, notoginseng treated samples. Data are representative of 2 separate experiments with 3 samples per treatment group.

Figure 4-3.

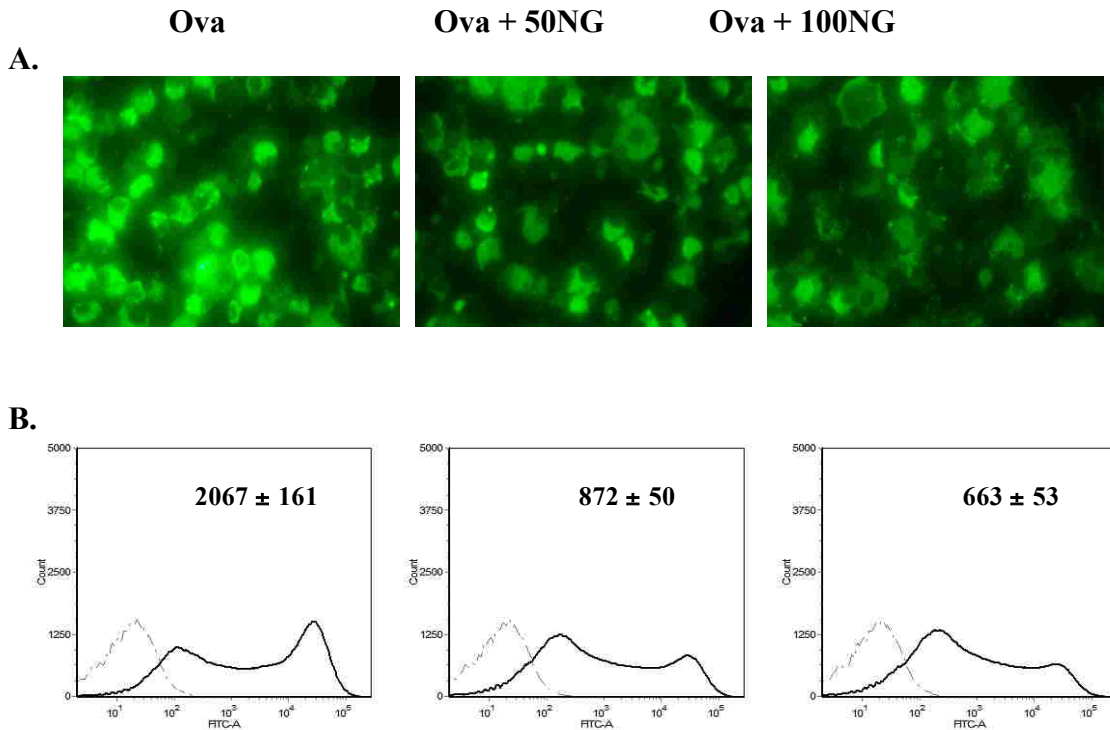


Figure 4-3. Notoginseng reduces FITC-conjugated ovalbumin uptake by BMDCs. Cells were incubated with FITC-conjugated whole ovalbumin (FITC-Ova) or FITC-Ova and notoginseng (NG) 200 μ g/ml. BMDCs were harvested and fluorescence intensity was visualized by fluorescence microscopy (A) and quantified by flow cytometry (B). In Fig. B, the dotted lines represent non-ova-exposed cells; solid lines represent ova exposed cells with or without notoginseng treatment. Data represents mean \pm SEM of 3 samples. * indicates significant differences between LPS-stimulated controls and LPS-stimulated, notoginseng-treated samples ($p < 0.05$). Data are representative of 2 separate experiments.

channel fluorescence intensity was detected between controls and cells concurrently exposed to 100 µg/ml notoginseng and FITC-ova (Fig. 4-3B).

NFκB activation in BMDCs is decreased by notoginseng treatment

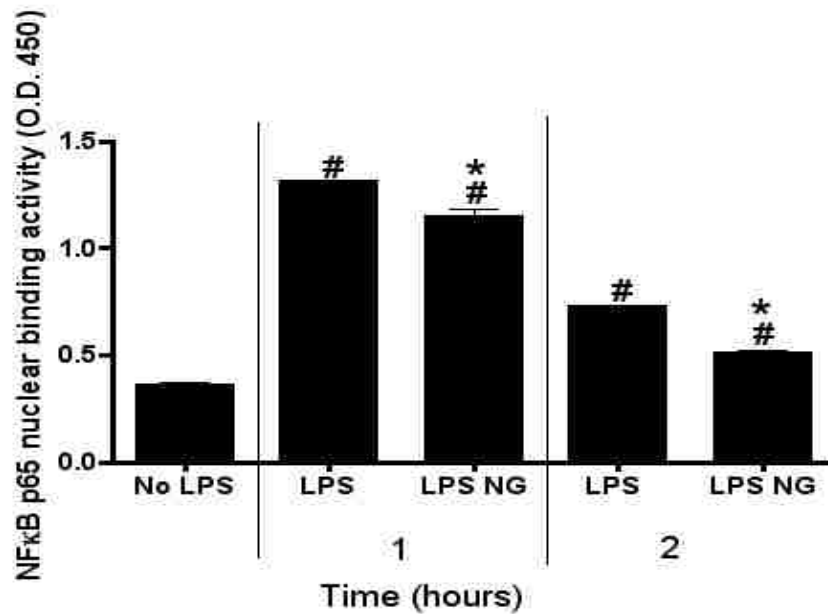
NFκB activation plays an important role in regulating the expression of many inflammatory molecules. To determine if the effects of notoginseng might be mediated through decreased NFκB activation, BMDCs were stimulated with LPS or LPS and 200 µg/ml of notoginseng for 0-2 hrs and NFκB p65 activity was assessed (Fig. 4-4). Nuclear extracts were evaluated to determine whether notoginseng altered NFκB p65-binding activity to an oligonucleotide containing the NFκB consensus-binding site. A 2.4-fold increase in NFκB binding activity was detected in the LPS-stimulated samples when compared to the unstimulated controls (Fig. 4-4A). Notoginseng treatment significantly decreased NFκB p65 binding activity in LPS-stimulated BMDCs. Reduced levels of nuclear NFκB p65 protein were also observed in notoginseng-treated BMDCs as determined by Western blotting (Fig. 4-4B).

Notoginseng does not inhibit BMDC activity via the glucocorticoid receptor

Previous reports have demonstrated binding and activation of the GCR by ginsenosides (Chung et al., 1998; Kim et al., 1997). Additionally, GCR activation leads to decreased inflammatory mediator production by DCs, similar to the effects of notoginseng. Therefore, the role of GCR activation was assessed in notoginseng-treated BMDCs. To determine whether notoginseng acts through GCR activation, LPS-stimulated BMDCs

Figure 4-4.

A.



B.

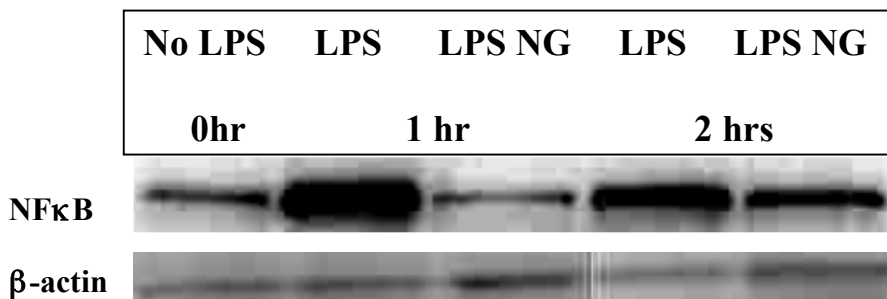


Figure 4-4. NFκB activation is reduced by notoginseng treatment. BMDCs were stimulated with LPS (1 μg/ml) and concomitantly treated with notoginseng (150 μg/ml). Cells were harvested after 2hrs and nuclear proteins were isolated. NFκB p65 activation was measured using the TransAM activity ELISA (A), and nuclear protein levels were determined using by Western blotting (B). # Indicates significant differences between LPS-stimulated and unstimulated cells ($p < 0.05$); * indicates significant differences between LPS-stimulated controls and LPS-stimulated, notoginseng-treated samples ($p < 0.05$). Data are representative of 3 separate experiments.

Figure 4-5.

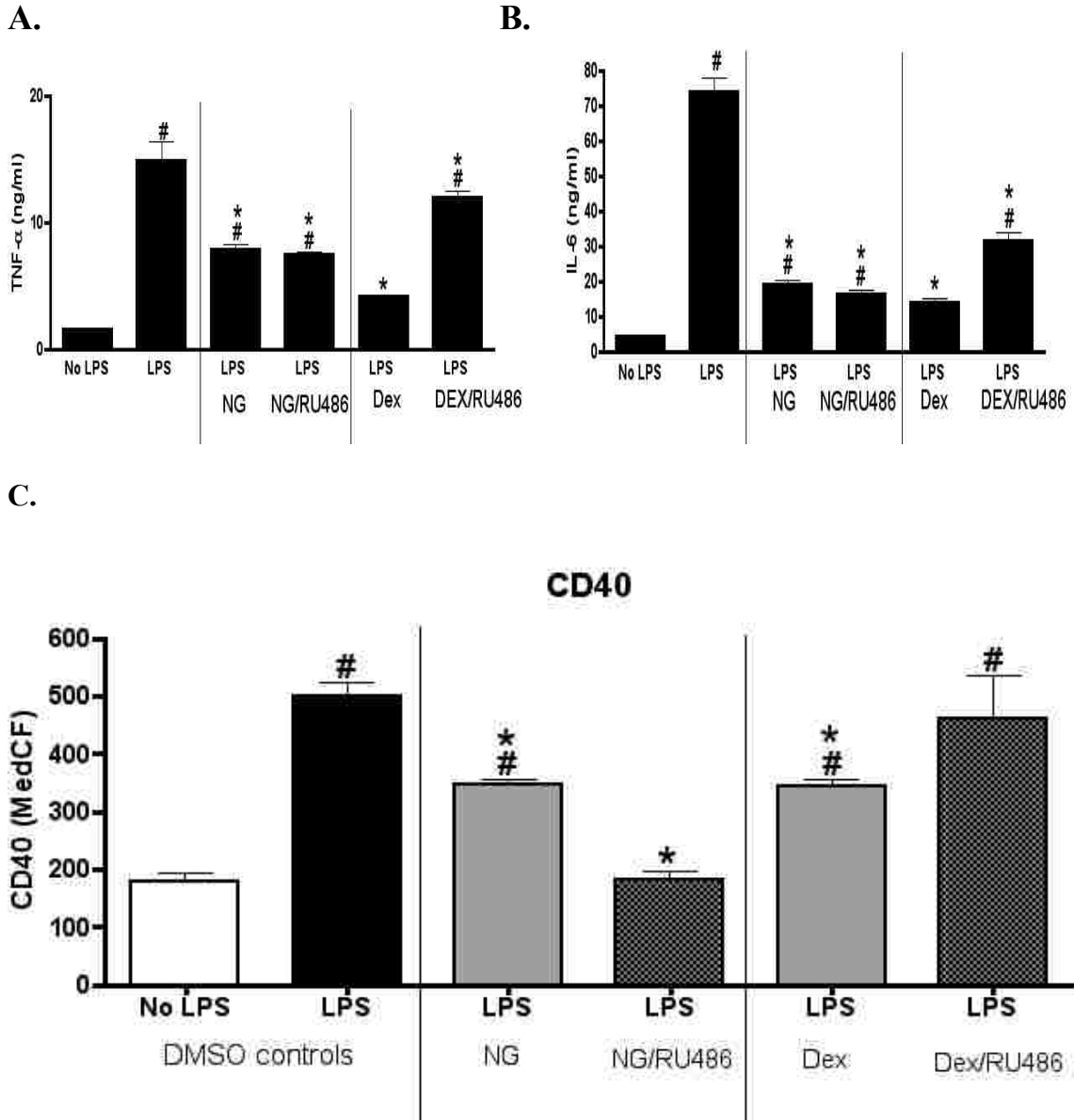


Figure 4-5. The effects of notoginseng on BMDCs are not mediated through the GCR. BMDCs were stimulated with LPS (1 μ g/ml) and concomitantly treated with notoginseng (200 μ g/ml) or dexamethasone. RU486 was added to some samples 15 minutes before addition of LPS, notoginseng or dexamethasone. Samples were harvested at 24 hours and TNF- α and IL-6 levels were measured by ELISA. CD40 expression was determined by flow cytometry. Data represents mean \pm SEM of 3 samples. # Indicates significant differences between LPS-stimulated and unstimulated cells ($p < 0.05$); * indicates significant differences between LPS-stimulated controls and LPS-stimulated, notoginseng-treated samples ($p < 0.05$). Data are representative of 2 separate experiments.

were either treated with 200 µg/ml of notoginseng, or 200 µg/ml of notoginseng and RU486. LPS-stimulated BMDCs were also treated with DEX and RU486 as comparative control as these compounds have been widely used to investigate whether the effects of a particular agent occurs via GCR binding (Kim et al., 2001; Pan et al., 2001). As expected, DEX significantly reduced BMDC production of TNF- α and IL-6, as well as their expression of CD40, with effects that were blocked with the co-administration of RU486 (Fig. 4-5). Notoginseng also significantly reduced TNF- α and IL-6 levels and CD40 expression. In contrast to DEX, however, RU486 did not reduce the inhibitory effects of notoginseng, as would be predicted if notoginseng acted through the GCR (Fig 4-5).

The effects of notoginseng treated BMDCs on antigen-specific T cell proliferation in vitro

A primary function of a DC is to uptake antigen and present it to T cells, culminating in the generation of a successful adaptive immune response. To investigate whether notoginseng affected Ag processing and presentation by DC, BMDC were treated with 200 µg/ml notoginseng for 24 hours and exposed to Ag in the form of ova peptide (OP) or whole ovalbumin (WO). Antigen-loaded BMDC were then cultured with ova-specific OTII T cells and T cell proliferation evaluated via [³H]-thymidine incorporation. A 3-fold and 9-fold increase in T cell proliferation was observed using OP- and WO- loaded BMDCs, respectively (Fig. 4-6A). While a trend towards decreased T cell proliferation was determined for cultures containing Ag-exposed, notoginseng-treated BMDCs, there were no significant effects when compared to controls. There was also no difference in IL-2 production between control and notoginseng-treated groups (Fig. 4-6B).

Figure 4-6.

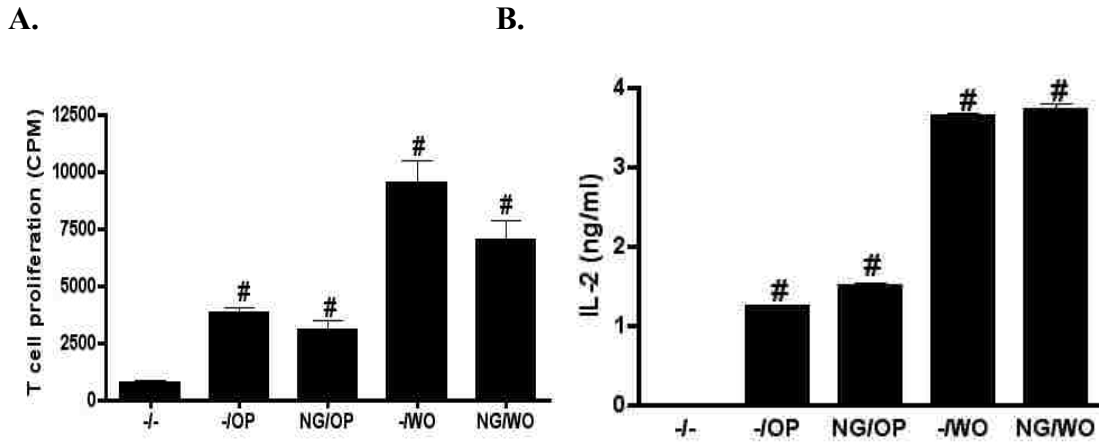


Figure 4-6. APC function of BMDCs is not affected by notoginseng *in vitro*. DCs were treated with notoginseng (200 $\mu\text{g/ml}$) for 24 hours after which the herbal extract was removed from the cultures. BMDCs were then stimulated with 100 $\mu\text{g/ml}$ of whole ovalbumin (WO) or ova peptide (OP) for an additional 24 hours. BMDCs were harvested and co-cultured with CD4⁺ T cells from OT II mice. After 3 days, T cell clonal expansion was measured as described in materials and methods (A) and supernatants analyzed for IL-2 production by ELISA (B). # Indicates significant differences between antigen exposed and unstimulated cells; * indicates significant differences between antigen-loaded controls and antigen loaded, notoginseng-treated samples ($p < 0.05$). Data are representative of 2 separate experiments; 3 samples per group.

Notoginseng does not affect BMDC migration or APC function in vivo

Immature DCs are capable of antigen capture in the periphery and subsequent migration to draining lymphatic tissue (Banchereau et al., 2000c). In lymph nodes, DCs activate antigen-specific T cells, thereby initiating T cell mediated immunity. To test the ability of notoginseng-treated DC to migrate to lymph nodes and interact with antigen specific T cells *in vivo*, donor BMDCs were treated with 200 µg/ml of notoginseng for 24 hours, and subsequently loaded with ova peptide. Congenic host mice that had been adoptively transferred with ova-specific CD4⁺ T cells were immunized with ova-loaded BMDCs. Mice were harvested 4 days following BMDC immunization and both the proximal and distal lymph nodes were removed for evaluation of BMDC-T cell interactions. Differences in the expression of cell surface molecules on donor and host mice, allowed for the segregation of donor DC (CD11c⁺/CD45.2⁺) and T cells (CD4⁺/Thy1.1⁺) from cells from the host mouse (CD45.1⁺/Thy 1.2⁺). Therefore, the effects of notoginseng treatment on BMDCs fate and function *in vivo* could be exclusively evaluated. Ag-loaded BMDCs increased percentages and numbers of OT II T cells in the draining popliteal lymph nodes of host mice when compared to the distal brachial lymph nodes which served as unactivated controls (data not shown). No significant differences were observed in the percentage or number of CD11c⁺ donor DCs present in the popliteal lymph nodes between control DCs and notoginseng-treated DCs (Figs. 4-7A and 4-7B). Additionally, there were no significant differences in the clonal expansion of OTII/Thy1.1 T cells in mice receiving control-treated and notoginseng-treated BMDCs (Fig. 4-7 C/D). Furthermore, no differences were observed in the activation of ova-specific

Figure 4-7.

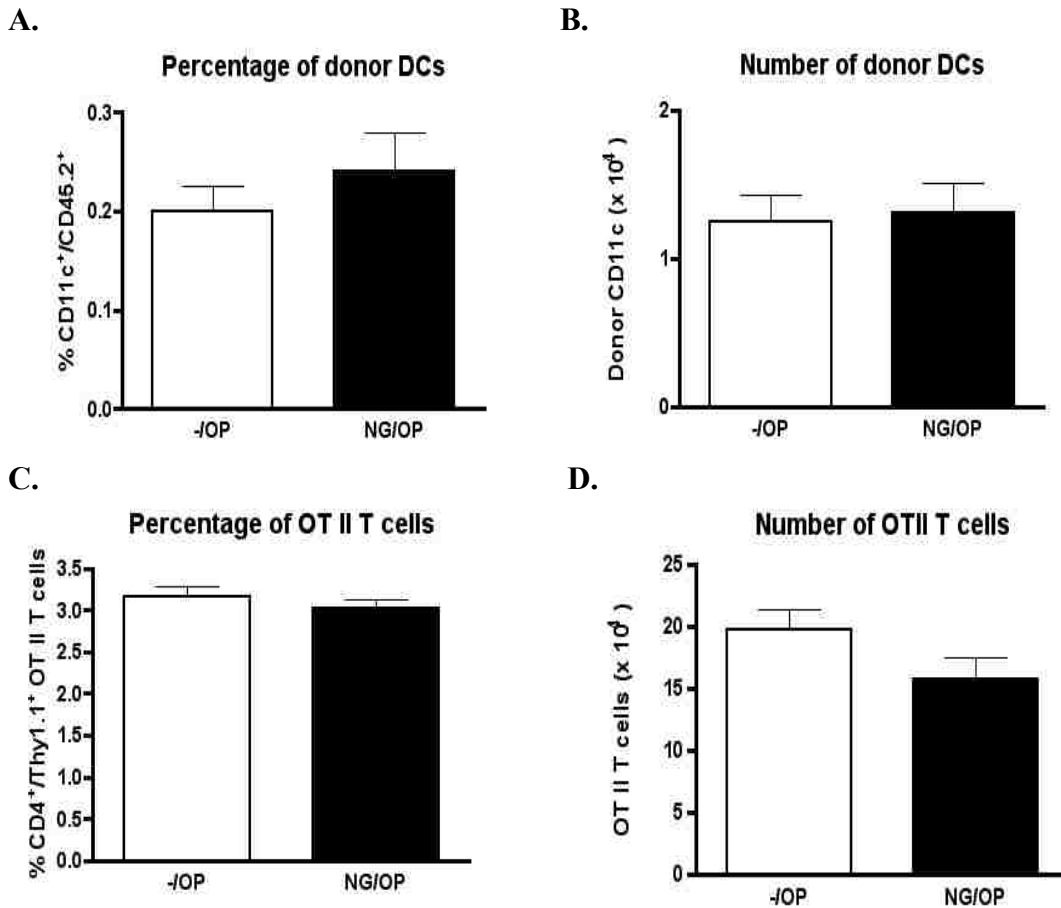


Figure 4-7. Notoginseng does not alter the ability of BMDCs to migrate to lymph nodes and activate T cells *in vivo*. CD4⁺ T cells from OTII/Thy1.1 were adoptively transferred into congenic CD45.1 host mice, 24 hours before immunization with ova-loaded BMDCs (OP) that were untreated (-) or exposed to notoginseng (NG) (200 µg/ml). On day 4 post-immunization, popliteal lymph node cells were harvested from host mice and analyzed by flow cytometry. Analysis was based on differences in phenotypic expression of cell surface molecules between donor DCs (CD45.2/CD11c) (A/B) and donor T cells (CD4/Thy1.1) (C/D) and cells from the host mice (CD45.1/Thy 1.2). Data are representative of 2 separate experiments, each with 6 animals per treatment group.

T cells between controls and notoginseng-treated cells as defined by the expression of the activation markers, CD62L and CD44, on the OT II/Thy 1.1 T cells (data not shown).

Discussion and conclusion

DCs provide critical functions for the effective defense against pathogens via both innate and adaptive immune responses. In this study we demonstrated that notoginseng decreased the level of pro-inflammatory cytokine production and cell surface molecule expression by dendritic cells without altering their T cell stimulatory capabilities.

Cytokines are soluble mediators that are involved in many biological processes in particular inflammation. TNF- α is a preformed cytokine and is released immediately after activation of phagocytes (Abass et al., 1994). In many inflammatory disease processes such as arthritis, TNF- α is described as being the apex of the pro-inflammatory cascade (Andreacos et al., 2004a). Inhibiting TNF- α has been shown to decrease production of other cytokines including IL-1 β , IL-6 and even TNF- α itself (Abass et al., 1994). Notoginseng reduced the secretion of TNF- α by BMDCs, but displayed an even greater inhibition of IL-1 β , IL-6 and IL-12. IL-10 production was also inhibited by notoginseng (data not shown). These data are consistent with previous results from our laboratory demonstrating that notoginseng significantly attenuates the production of TNF- α and IL-6 by LPS-stimulated murine macrophages (Rhule et al., 2006b). DCs have increasingly been shown to participate in inflammatory disease processes. As such, inhibition of pro-inflammatory cytokines suggests a plausible mechanism for the described anti-inflammatory effects of notoginseng (Jin et al., 2007; Li and Chu, 1999; Wang et al., 2006b).

Accessory molecule expression is necessary for full T cell activation by DCs. The interaction of CD40 on DCs with CD154 (CD40L) on T cells is crucial in sustaining a productive T cell response (Banchereau et al., 2000b). Additionally, CD40 is involved in the amplification and regulation of inflammatory responses (van Kooten and Banchereau, 2000). In our experiments, CD40 expression was decreased following notoginseng treatment of BMDCs, while expression of CD86 and MHC II expression was less sensitive. These results suggest that notoginseng may inhibit the expression of cell surface molecules via different mechanisms, but it is particularly effective in decreasing CD40 expression. One possible explanation for the selective effects of notoginseng on CD40 is through decreased activation of NF κ B, especially the RelB. Interestingly, RelB knockout mice lack CD40 expression, yet express normal levels of CD86 and MHC II (Martin et al., 2003; O'Sullivan and Thomas, 2003). It is therefore plausible that notoginseng alters CD40 expression on DCs via modulation of RelB; however, this possibility remains to be tested.

Notoginseng significantly reduced BMDC cytokine production and expression of CD40, effects that can promote T cell tolerance (O'Sullivan and Thomas, 2003). There was also a decrease in phagocytosis, which is consistent with a report on the effects of metabolized ginsenosides on human DCs by Takei and colleagues (Takei et al., 2004). Several reports have described the inhibitory effects of ginseng on NF κ B p65 activity (Ahn et al., 2006; Oh et al., 2004a; Park et al., 2005). As many DC functions are mediated through NF κ B p65 signaling, the effects of notoginseng on this protein were examined in BMDCs. In our study, notoginseng significantly decreased NF κ B p65 nuclear levels and activity in BMDCs. Recent studies suggest that the RelA subunit of

NF κ B is crucial for the expression of inflammatory cytokine genes, but not T cell stimulatory genes such as CD86 and MHC II (Wang et al., 2007). Alternatively, notoginseng could be selectively affecting different NF κ B components such as RelB, or other signaling pathways such as MAP kinase and AP-1 as recently demonstrated in astroglial cells (Jung et al., 2006). Additional experiments focused on defining the effects of notoginseng on intracellular signaling events are therefore warranted.

Glucocorticoids are hormones that are naturally released during the initiation and regulation of an immune response. These hormones activate the GCR thereby reducing the synthesis of inflammatory mediators via repression of gene transcription (Smoak and Cidlowski, 2004). Previous studies in FTO2B cells, a rat hepatoma-derived cell line, showed that the ginsenoside Rg1, a component of ginseng, can effectively activate the glucocorticoid receptor (Chung et al., 1998; Lee et al., 1997). In our study, RU486 completely inhibited the effects of DEX on CD40. However, the effects on TNF- α and IL-6 were not completely reversed. Blockade of the GCR with the antagonist RU486 failed to reduce the inhibitory effects of notoginseng on BMDCs. These results are consistent with a previous study showing that ginseng does not mediate its effects via GCR activation, but reverses the down-regulation of the activated GCR (Ling et al., 2005). Additionally, as we previously reported that, individual ginsenosides such as Rg1 can differentially affect APCs in the presence of other ginsenosides such as Rb1 (Rhule et al., 2006b).

Alternatively, notoginseng may indirectly affect GCR activity by binding to locations other than the active site. If binding of notoginseng did not occur via the active site of the GCR in BMDCs, then RU486 would not affect its activity in BMDCs. Unexpectedly, the

combined treatment of LPS-stimulated BMDCs with RU486 and notoginseng resulted in further decreases in the expression of CD40. While we have not directly studied what caused this effect, as RU486 can also block glucocorticoid and progesterone receptor-binding sites, there may be more notoginseng available for activity at other complexes. This may have led to more unbound notoginseng being available to interact with other receptors responsible for modulating the immune system, resulting in the more pronounced decrease of CD40 observed with the addition of both RU486/notoginseng.

DCs are the primary APCs involved in the initiation of T cell activation. Notoginseng treatment reduced cytokine production, phagocytosis and costimulatory molecule expression by BMDCs. As alterations of these DC functions can directly affect their activation of T cells, we examined the effects of notoginseng on APC activity. Notoginseng treatment did not affect the ability of BMDCs to stimulate antigen specific T cell proliferation *in vitro* and *in vivo*. There were also no effects on activation molecule expression on T cells interacting with notoginseng-treated BMDCs. Our results are inconsistent with other studies suggesting that notoginseng and its components possess adjuvant properties (Qin et al., 2006; Sun et al., 2005; Yang et al., 2007a). This discrepancy could be due to several factors including the ratios of ginsenosides in our notoginseng extract being different from those used in other experiments. This explanation is in line with previous experiments by our laboratory demonstrating that ginsenoside Rb1 and Rg1 can have different properties in regards to proinflammatory cytokine inhibition induced by LPS in RAW264.7 and DC2.4 cells (Rhule et al., 2006b)(submitted article). Additionally, in a study by Cho et al ginsenosides were shown

to differentially regulate lymphocyte proliferation *in vitro* (Cho et al., 2002b). This demonstrates the potential for different ginsenosides to possess varied effects.

DCs *in vivo* had decreased CD40 expression at the end of the 4 day incubation period with T cells, while CD86 and MHCII were expressed at similar levels on the untreated and notoginseng-treated DCs (data not shown). Importantly, the decreased CD40 expression on DCs clearly had no effect on the activation of T cells. This is significant, as interaction of CD40 on APCs with CD40L on T cells is necessary for full T cell activation (Fujji et al., 2004). Our experiments therefore raise questions of how much CD40 expression is necessary to fully activate T cells and whether decreased expression leads to immunosuppression. There are several studies describing the effects of Ginseng on T cell proliferation and reduced function/activation of APCs such as macrophages (Cho et al., 2002b; Martin et al., 2003; Oh et al., 2004a; Rhule et al., 2006a). However, the effects of notoginseng on the interactions of APCs with antigen-specific T cells have not been determined. Our experiments provide novel information, demonstrating that although innate functions of DCs are decreased by notoginseng, adaptive immune functions are unaffected.

There is continued interest in the effects of natural products due to their therapeutic potential. Our study demonstrates that notoginseng treatment of BMDC can inhibit specific mediators of inflammation, including pro-inflammatory cytokine production and CD40 expression, to a similar degree as dexamethasone, without the immunosuppressive effects on T cell mediated immunity that is normally associated with this drug. Taken together, our research suggests that notoginseng possesses anti-inflammatory effects without significantly affecting Ag-specific interactions between DCs and T cells. Thus,

notoginseng may be an effective natural treatment for inflammatory diseases such as arthritis, inflammatory bowel syndrome and asthma.

Acknowledgments

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CHAPTER 5.

An assessment of the immunotoxicological effects of *Panax notoginseng* in ovalbumin immunized mice.

Unpublished Data

Abstract

Many herbals have a long history of use and health claims that have not been confirmed by scientific studies. As a result, there are numerous cases in which natural products not only do not confer suggested benefits, but also cause harmful effects. There are a number of studies characterizing the general toxicity of the popular herbal ginseng, but none that was formulated specifically to examine whether ingestion of this natural product can generate immunotoxicity. In this regard, the effects of high dose and low dose *Panax notoginseng* treatment was studied in ovalbumin/alum immunized C57BL/6 and Balb/c mice, respectively. Mice were gavaged for 10 days with either a high dose (1g/kg) or low dose (660 µg/kg) notoginseng or PBS as control. On day 4 of notoginseng treatment, mice were immunized with 100 µl of a mixture containing 20 mg ovalbumin and 2 mg alum by i.p. injection. C57BL/6 and Balb/c mice were harvested 14 days and 12 days post immunization, respectively. No effects on body, spleen and thymic weights were observed between high or low dose notoginseng-treated and control groups. However, there was a decrease in cell numbers in the mesenteric lymph nodes with high dose notoginseng treatment. Additionally, ova-specific IgM levels were decreased after 7 days of exposure to the high dose of notoginseng. Conversely, there were no effects on ova-specific IgG levels on day 7 post-immunization. There were also no effects on IgG or IgM levels at the later time point in either the high or low dose treatment groups. High doses of notoginseng also decreased the ability of spleen cells to produce TNF- α after restimulation with LPS *ex vivo*. Taken together, our results suggest that there is limited immunotoxicity associated with high dose notoginseng exposure. However, this toxicity is not present with low dose notoginseng treatment.

Introduction

The roots of the *Panax notoginseng* plant have been traditionally used in Chinese medicine because of their potential to treat a variety of ailments including inflammation, cardiovascular diseases and cancer. Recently, there has been an increase in the use of this plant and other herbals in Western countries including the US. The popularity of Ginseng and other natural products is thought to be on the rise because of factors such as increased availability over the Internet and other sources. Other reasons include an aging population and experimenting with alternative treatment to alleviate symptoms of chronic disease (Kinsel and Straus, 2003).

The safety and efficacy of many herbals have not been established. This is primarily due to the 1994 Dietary Supplement Health and Education Act which classifies natural products as foods. There are few studies characterizing the clinical efficacy of Ginseng on a variety of conditions including diabetes and cancer (Carabin et al., 2000). However, many of these studies are limited by the apparent differences in pharmacological effects noted between Ginseng species (Kitts and Hu, 2000). Some studies suggest that there is a low incidence of toxicity associated with ginseng intake (Carabin et al., 2000). There have also been cases where Ginseng overdoses have been reported. This condition is referred to as “ginseng abuse syndrome”. Nonetheless, there is some confusion as to the validity of such a syndrome. This confusion is primarily caused by the lack of control or analysis for the identification of the ginseng taken, or whether it was actually Ginseng or another supplement that caused the toxicities in these patients (Carabin et al., 2000; Kitts and Hu, 2000).

The primary biologically active components of notoginseng are thought to be the ginsenosides (Hasegawa, 2004). After oral ingestion these ginsenosides can pass through the stomach without decomposition but are deglycosylated by colonic bacteria in the large intestines prior to absorption. Following absorption, metabolites can be further esterified with fatty acids by the liver to form additional bioactive derivatives. Studies using rats suggest that the bioavailability of the intact ginsenosides is very low varying from 0.1-18 percent.

Alterations in immune function can lead to several conditions, including increased incidence of hypersensitivity disorders, autoimmune and infectious diseases or neoplasia (Germolec, 2004; Selgrade, 1999). This change in immune function can result from several synthetic or natural agents that can cause injury or insult to the immune system (Delaney et al., 2001; Germolec, 2004). These agents are classified as immunotoxicants, and are of considerable public health concern (Germolec, 2004). Potential immunotoxicants are usually identified by using a variety of strategies to screen for their harmful effects on the immune system. Tests include screening for changes in immune organ weights, serum immunoglobulin levels and immune cell numbers (Germolec, 2004). Additional analyses include the quantitation of cell surface markers by flow cytometry and cytokine production in immune cells in response to stimulation or challenge (Germolec, 2004).

Notoginseng has diverse immunomodulatory properties including reducing inflammation and boosting adjuvant activity (Sun et al., 2004). However, none of these studies have formally explored the possible immunotoxicities that could arise from notoginseng treatment. In this regard, the immunotoxicological effects of *Panax*

notoginseng were examined using ovalbumin immunized Balb/c mice. Balb/c mice were gavaged with a high or low dose of notoginseng or PBS controls and immunized with ova/alum. During the treatment period, weight changes of the mice were assessed daily as a measure of general toxicity between treatment and control groups. Mice were harvested 10 days post immunization and the weight and cell numbers, as well as cell surface molecule expression of immune organs were examined for differences between notoginseng and control groups. Spleen cells were stimulated with LPS ex vivo to determine if notoginseng altered the immune responsiveness of these cells to an inflammatory agent. Antigen specific antibody levels were also characterized in serum collected from mice before immunization and immediately after harvesting. Our study demonstrates that there is limited immunotoxicity associated with notoginseng intake.

Material and Methods

Animals

Two- to four- month old male C57BL/6 and Balb/c mice were bred and maintained in the animal research facilities at the University of Montana. Mice were housed under specific pathogen-free conditions and maintained on a 12-hour dark/light cycles. Standard laboratory food and water were provided ad libitum. All protocols for the use of animals were approved by the University of Montana Institutional Animal Care and Use Committee.

Chemicals

Extracts were obtained from the roots of the *Panax notoginseng* (Burk.) F.H. Chen ex C.Y. Wu & K.M. Feng plant using ethanol and standardized to contain Rb1 and Rg1

ginsenosides at 35 and 34% of the whole extract, respectively. This resulting Noto-GTM extract was kindly supplied by Technical Sourcing International, Inc. (TSI, Missoula, MT). Levels of Rb1 and Rg1 in the notoginseng extract were determined by high-performance liquid chromatography analysis by TSI. Certification of analyses was approved by Xia Ronglong (QA manager, TSI). There were no detectable amounts of *Escherichia coli* (E. coli) or *Salmonella enterica* in the notoginseng preparation (unpublished data). The extract was dissolved in complete media (see below) and subsequently sterile-filtered through a 0.22 µm Millipore membrane. The Lipopolysaccharide (LPS) from E. coli (055:B5) was obtained from Sigma-Aldrich.

Immunization

Balb/c mice were gavaged once daily for 10 consecutive days with either PBS (vehicle) or a low dose (660 µg/kg) of notoginseng which was equivalent to concentrations which the average person ingesting ginseng supplements would be exposed to. In a separate experiment, C57BL/6 mice were gavaged with PBS controls or a high dose (1mg/kg) of notoginseng. On day 4 of treatment, both BALb/c and C57BL/6 mice were immunized with 100 µl of a mixture containing 20 mg ovalbumin and 2 mg alum (ova/alum) via I.P. injections. Balb/c mice were harvested 11 days post immunization, (15-days after notoginseng treatment), while C57BL/6 mice were harvested 14 days post immunization. The thymuses, spleens, mesenteric (MLNs), popliteal (PLNs) and brachial (BLNs) lymph nodes were removed from the animals for immunotoxicological analysis. Serum was also collected from each mouse and used for measurement of antibody titers.

Organ weights and cell counts

The spleens and thymuses were harvested and weighed for both the high dose and low dose notoginseng treated groups. The spleens, MLN, PLN and BLN were processed and cell numbers were calculated using a Coulter counter.

Preparation of spleen and lymph node cells

Single cell suspensions were prepared by pressing spleens or lymph nodes through cell strainers using the tops of 1 ml syringes. Erythrocytes were removed from spleen cell suspension by hypotonic lysis. Spleen and lymph node cells were then washed and resuspended in RPMI (GibcoBRL, Grand Island, N.Y), supplemented with 10% FBS (Hyclone, Logan, UT), 50 μ M mercaptoethanol, 20 mM HEPES, 10 mM sodium pyruvate and 50 μ g/ml gentamicin (GibcoBRL, Grand Island, N.Y).

Flow cytometry

Detection of cell surface molecule expression on spleen and lymph node cells treated with low dose notoginseng was analyzed by fluorescent activated cell sorting (FACS) as previously described (Shepherd et al., 2001). Briefly, cells were harvested and washed with PAB (1% bovine serum albumin and 0.1% sodium azide in PBS). Cells were blocked with 30 μ g of purified rat and/or hamster IgG (Jackson ImmunoResearch, West Grove, PA) for 10 minutes to eliminate non-specific staining. Optimal concentrations of fluochrome-conjugated murine monoclonal antibodies were used to stain cells for an additional 10 minutes. The antibodies used in these experiments were CD4⁺, CD8⁺, CD11b, CD11c, CD19, and their corresponding isotype controls (BDPharmingen, San Diego, CA). One to five hundred thousand viable cells per treatment (as determined by

light scatter profiles) were analyzed using a BD FACSAria flow cytometer and FACSDiva software (BD Biosciences, San Jose, CA).

Measurement of OVA-specific antibody

OVA-specific antibodies for total IgG in serum were detected by indirect ELISA. Briefly, 96-well plates were coated with 100 µg/ml whole ovalbumin (diluted in dPBS) overnight at 4 °C. The wells were washed three times with PBS containing 0.05% (v/v) Tween 20 (PBST), and blocked with 3% BSA/PBS for 2 hrs. Serum samples were added at 1:10-10⁶ sequentially diluted samples. BSA/PBS (1%) was used as controls. The plates were then incubated for 2 hrs. Aliquots of IgG (1:4000) and IgM (1:250) horseradish peroxidase conjugate antibodies were added to each plate for an additional 1 hr. The plates were then developed and the optical density (OD) was measured in a spectrophotometer reader at 450 nm.

Assay for cytokine production by spleen cells ex vivo

Spleen cells were incubated in RPMI (GibcoBRL, Grand Island, N.Y), supplemented with 10% FBS (Hyclone, Logan, UT), 50 µM mercaptoethanol, 20 mM HEPES, 10 mM sodium pyruvate and 50 µg/ml gentamicin (GibcoBRL, Grand Island, N.Y). Cell were stimulated with LPS and harvested after 24 hrs. Supernatants were collected from the LPS stimulated samples and the production of IL-6 and TNF-α were measured by enzyme-linked immunosorbent assay (ELISA). Samples were analyzed according to the manufacturer's recommendations with mouse cytokine-specific BD OptEIA ELISA kits (BD PharMingen, San Diego, CA).

Results

Notoginseng treatment has no effects on the weights of mice

Biological processes, including the metabolism of chemicals, in humans and laboratory animals are related to body weight (Huggett et al., 1996). As such, we first identified the toxicity of notoginseng using changes in the weight of C57BL/6 (Fig. 5-1A) or Balb/c (Fig. 5-1B) mice. Animals were gavaged with either a high or low dose of notoginseng over a ten-day period (Fig. 5-1). Although there was the expected weight loss in both control and notoginseng group immediately after immunization, there were no significant changes in the weights of animals with either high or low dose notoginseng treatment when compared to PBS control groups.

The effects of notoginseng on immune tissues

Alterations in the weight or cell number of immune tissue generally denote gain or loss of cells by either changes in proliferation or cell viability. In these experiments, there were no differences in organ weights of the spleens and thymuses in mice treated with either high or low dose notoginseng (Fig. 5-2A/C). However, there was a decrease in the cell numbers in the mesenteric lymph nodes in mice treated with a high dose of notoginseng (Fig. 5-2B). Conversely, spleen, PLN and BLN cell numbers were unchanged with high dose notoginseng exposure (Fig. 5-2B). No differences in cell numbers were observed in any of the tissues tested after low notoginseng exposure (Fig. 5-2D).

Notoginseng differentially alters antigen specific antibody levels in Balb/c mice

The primary function of the immune system is to protect the body against pathogens.

Figure 5-1.

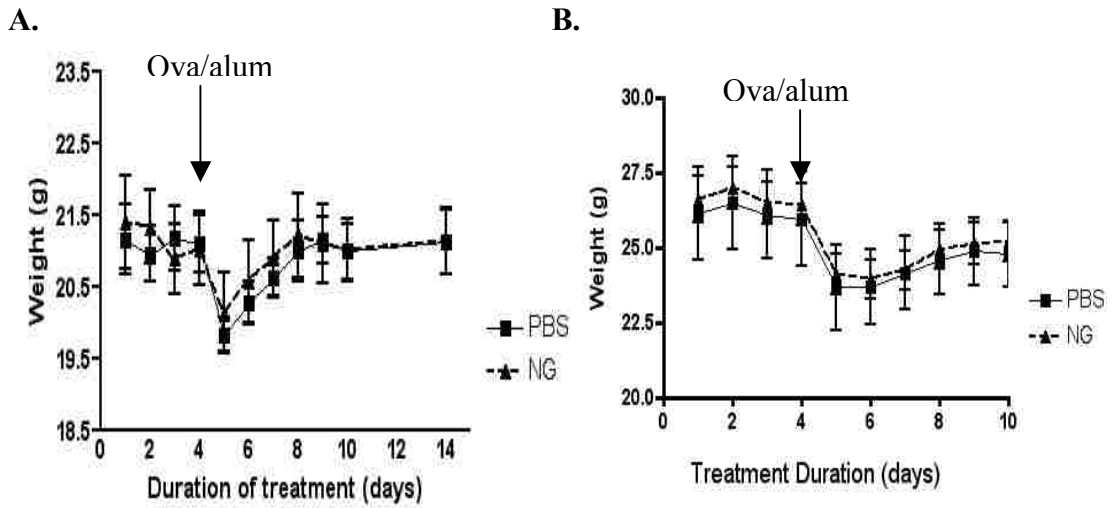


Figure 5-1. The body weights of mice are unchanged by notoginseng (NG) treatment. (A) C57BL/6 and (B) Balb/c mice were gavaged with low and high dose notoginseng, respectively, or PBS controls. At day 4 of treatment mice were immunized with ova/alum as previously described in the materials and methods section. Mice were assessed daily for changes in body weight, and general overall health. Error bars indicate mean \pm SEM of 5 C57BL/6 and 6 mice Balb/c mice.

Figure 5-2.

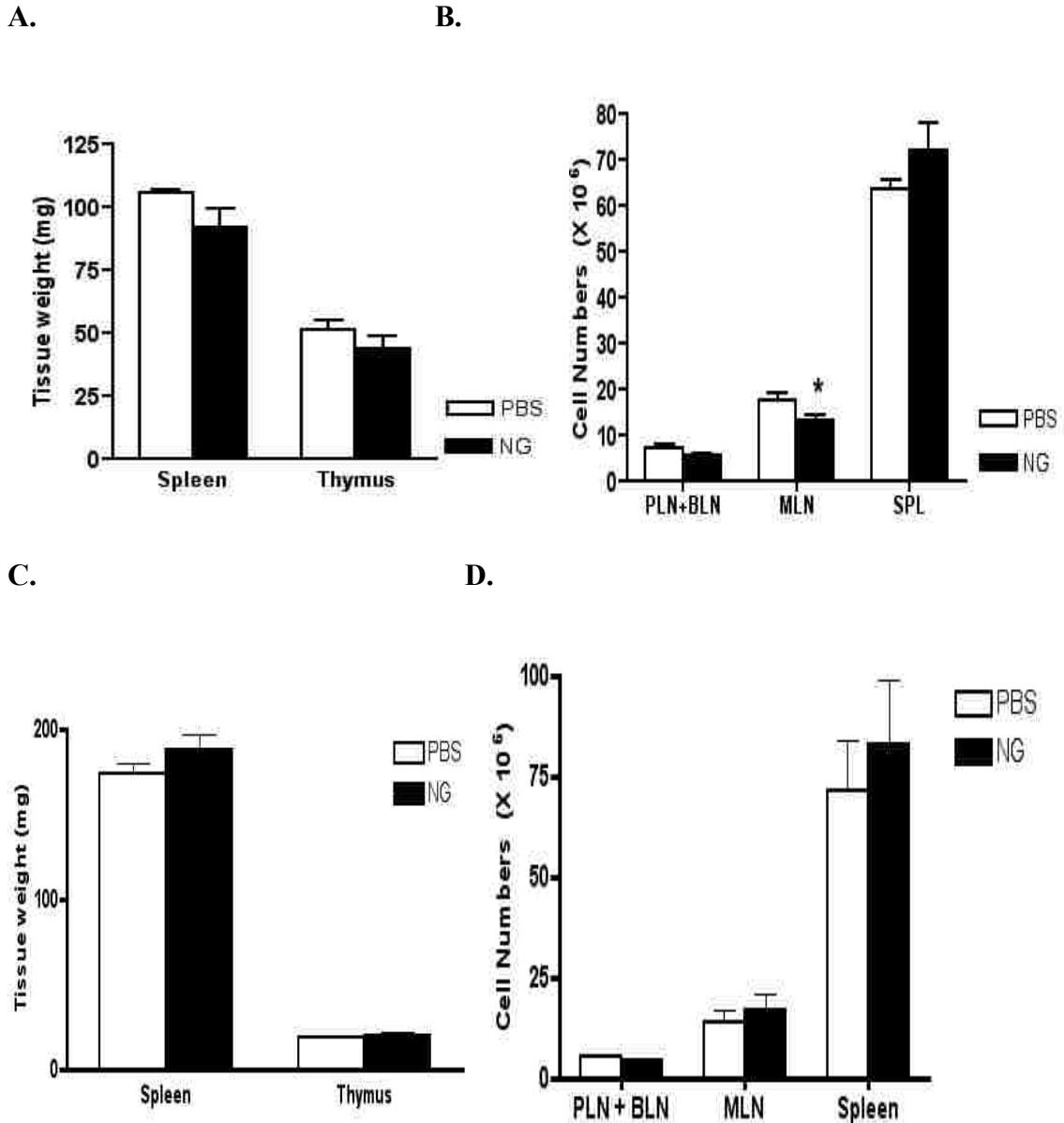


Figure 5-2. Notoginseng (NG) selectively affects cell numbers, but does not alter the weight of immune organs in C57BL/6 and Balb/c mice. Mice were gavaged with low (A,B) and high (C,D) dose notoginseng, or PBS controls and immunized with ova/alum as previously described in the materials and methods section. At the end of the experiment period, immune organs were harvested and analyzed for changes in weight, and numbers. Error bars indicate mean ± SEM of 5 C57BL/6 and 6 mice Balb/c mice; *, $p < 0.05$ for the comparison of PBS and notoginseng treated mice.

After exposure to antigen, the innate immune system stimulates adaptive immune cells to attack foreign antigens. In these experiments, mice that were exposed to notoginseng or PBS were immunized with the adjuvant ova/alum to simulate exposure to a pathogen and induce B cells to produce antigen specific antibody responses. Serum was collected from mice gavaged with high dose notoginseng on days 7 and 17 after exposure (Table 5-1). There was a 27 % decrease in IgM levels on day 7 of high dose notoginseng treatment. However, there was no significant difference in IgG or IgM in either the control or notoginseng treated group on day 17 of the experiment.

In Balb/c mice treated with a low dose of notoginseng, serum was collected only at the end of the experiment period (day 15) (Table 5-2). There was no significant difference between notoginseng and control treated mice in ova-specific IgG and IgM with low dose exposure.

Notoginseng exposure elicits dose dependent differences in the sensitivity of splenocytes to LPS stimulation ex vivo

The spleen is the largest secondary lymphoid organ and contains a variety of immune cells including B cells and T cells, in addition to the APCs macrophages and DCs (Cesta, 2006; Elmore, 2006). Most of these cells respond to a stimulus such as LPS by a number of mechanisms including the production of cytokines. As such, we examined the effects of notoginseng on the response of spleen cells to the bacterial component, LPS. Spleen cells from PBS controls and notoginseng treated groups were stimulated with 1 µg/ml LPS for 24 hrs. As shown in Fig. 5-3A, TNF-α production was reduced by 25 % in the high dose notoginseng treated group as compared to PBS control. There was also a

Table 5-1. High Dose notoginseng treatment differentially affects antigen-specific antibody generation *in vivo*

Antibody Type	DAY 7 post initial NG exposure		DAY 17 post initial NG exposure	
	PBS ^a	Notoginseng	PBS	Notoginseng
IgG ^b	0.58 ± 0.2	0.38 ± 0.15	0.58 ± 0.02	0.62 ± 0.07
IgM	0.36 ± 0.02	0.26 ± 0.04*	0.54 ± 0.25	0.28 ± 0.10

^a C57BL/6 mice were gavaged with a PBS controls or a high dose of notoginseng (NG) (1g/kg) for 10 days. At 4-days of treatment mice were immunized with ova/alum by I.P injections. Sera were collected from mice at 7 days and 17 days after notoginseng exposure (3-days and 14 days post immunization, respectively).

^b Optical density (O.D.) for serum antibody levels as determined by ELISA.

^c Error bars indicate mean ± SEM of 5 mice; *, $p < 0.05$ for the comparison of PBS and notoginseng treated mice.

Table 5-2. The effects of notoginseng on ova-specific antibody production in Balb/c mice

DAY 14 post initial exposure to NG		
Antibody Type	PBS	Notoginseng
O.D IgG	0.40 ± 0.16	0.34 ± 0.12
O.D IgM	0.40 ± 0.08	0.33 ± 0.06

^a Balb/c mice were gavaged with a low dose of notoginseng (NG) (660 µg/kg) for 10 days. At 4-days of notoginseng treatment mice were immunized with ova/alum by I.P injections. Sera were collected from mice at 14 days after notoginseng exposure (11 days post immunization).

^b Optical density (O.D.) for serum antibody levels as determined by ELISA.

^c Error bars indicate mean ± SEM of 6 mice.

Figure 5-3.

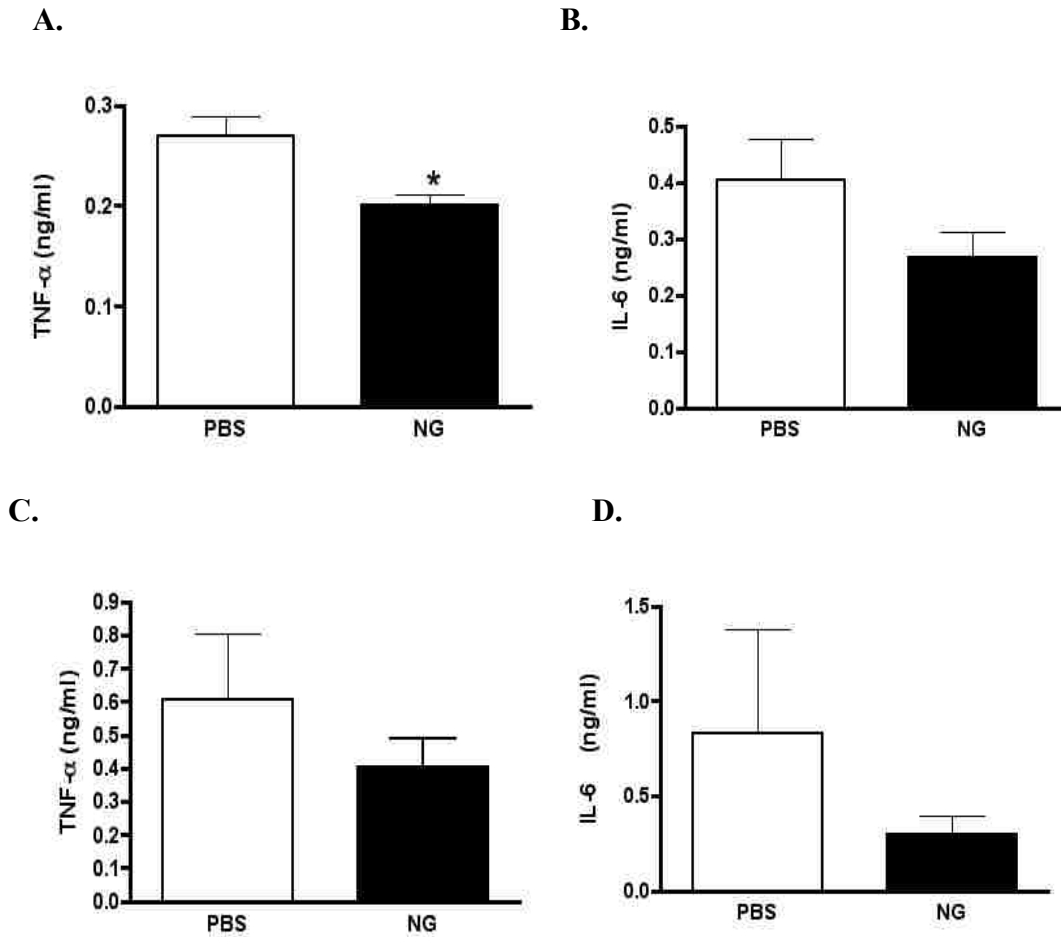


Figure 5-3. TNF- α production by LPS stimulated spleen cells is reduced by high dose notoginseng (NG) exposure. (A, B) C57BL/6 and (C, D) Balb/c mice were gavaged with low or high dose notoginseng, or PBS controls and immunized with ova/alum as previously described in the materials and methods section. Cells from the spleens of control and notoginseng treated mice were exposed to 1 μ g/ml of LPS for 24 hours. Supernatants were harvested and analyzed for TNF- α and IL-6 production by ELISA. Error bars indicate mean \pm SEM of 5 C57BL/6 mice and 6 Balb/c mice; *, $p < 0.05$ for the comparison of PBS and notoginseng treated mice.

trend towards decreased TNF- α production with spleen cells obtained from mice treated with low dose notoginseng. This decreased trend was also observed with IL-6 production with both high and low dose notoginseng.

The percentages of T cells, B cells, DCs and macrophages in immune tissues are not altered by low dose notoginseng treatment

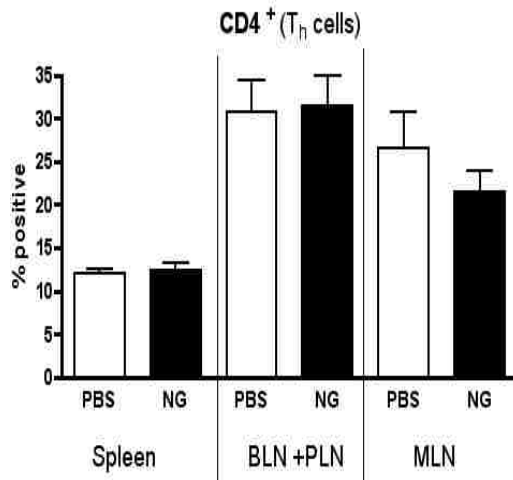
DCs and macrophages are important antigen presenting cells for initiating the activation of T and B cells thereby eliciting adaptive immune response. To determine whether there were any alterations in any of these populations by notoginseng treatment, cells were obtained from the spleen, PBLN and MLN and stained for lineage marker cell surface expression. There were no differences in the populations of DCs (CD11c⁺), macrophages (CD11c⁻/CD11b⁺), T_h cells (CD4⁺), T_c cells (CD8⁺) or B cells (CD19⁺) with notoginseng treatment as compared to PBS control groups (Fig. 5-4).

Discussion and conclusion

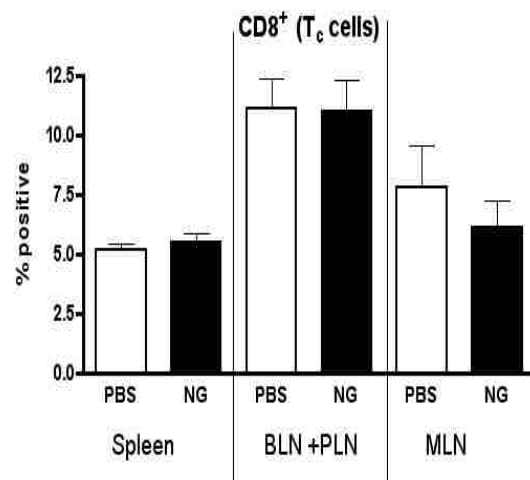
Immunotoxicology is the study of the adverse effects of natural and synthetic compounds on the immune system. Exposure to an immunotoxicant can result in immune dysfunction with outcomes including immunosuppression, or alternatively, allergy, autoimmunity or a number of other inflammatory based diseases. Therefore, identification of immunotoxicants is essential to maintaining homeostasis in an organism because of the critical role the immune system plays in sustaining host resistance to microbes. In this study, we evaluated the potential for notoginseng to elicit immunotoxicities in mice. As chemicals can generate general toxicities if they affect multiple organs, we examined weight changes in the tested animals throughout our experiment period.

Figure 5-4.

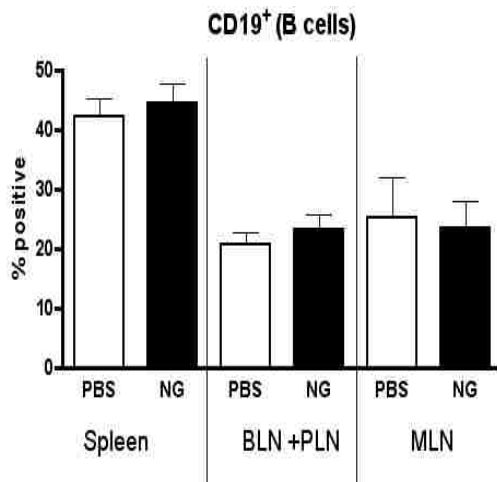
A.



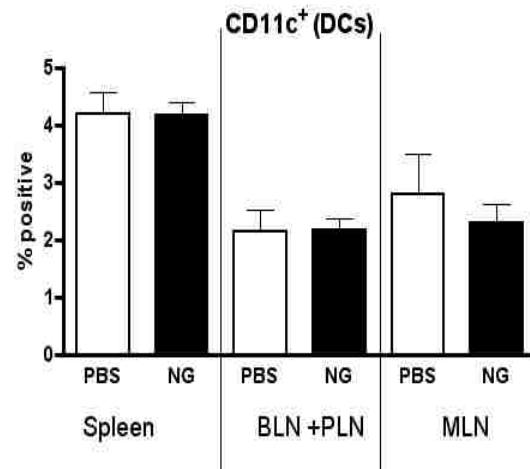
B.



C.



D.



E.

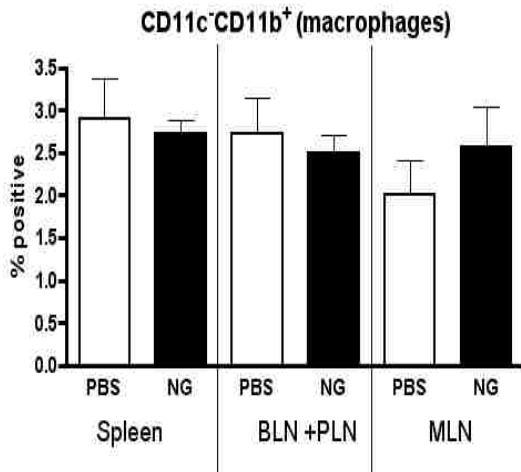


Figure 5-4. Low dose notoginseng (NG) treatment does not alter the percentages of APCs and T cells in the spleen and lymph nodes. Balb/c mice were gavaged with low dose notoginseng, or PBS controls and immunized with ova/alum as previously described in the materials and methods section. Based on lineage markers expression, cells from the spleen, MLN and PBLN were analyzed for changes in the percentages of T_h cells (CD4⁺), T cytotoxic cells (CD8⁺), B cells (CD19⁺), dendritic cells (CD11c⁺) and macrophages (CD11c⁻/CD11b⁺) by flow cytometry. Error bars indicate mean \pm SEM of 6 Balb/c mice.

There were no changes in the weights of the mice at both concentrations of notoginseng tested over a ten-day period, which is a good indication that acute doses of notoginseng do not induce overt toxicity. Our results with notoginseng are in line with previous studies that the LD₅₀ for *Panax ginseng* in mice as high as 5g/kg for oral administration (Carabin et al., 2000; Kitts and Hu, 2000).

Standard immunotoxicological tier testing involves assessing the weight of immune organs after exposure to a potential immunotoxicant (Burns-Naas et al., 2001). The thymus is one of the primary organs of the immune system. It is involved in “educating” T cells for recognition of self and non-self antigens. The spleen is a secondary immune organ, with the primary function of filtering the blood and removing both foreign antigens and circulating dead cells. Both immune organs are therefore important for optimal immune responses, and have been widely researched for toxicants which can impair their functions. No changes in the weight of spleen or thymic organs were observed at either dose of notoginseng examined. Changes in the weight of these organs usually occur because of edema or an alteration in the proliferation of immune cell in response to an immunotoxicant. As no differences were observed between treated and control groups, we concluded that notoginseng does not alter the number of cells present in these organs.

Lymph nodes are part of an immune network that filters antigens from interstitial lymphatic fluid (Burns-Naas et al., 2001). The mesenteric lymph nodes are a part of the gut associated lymphoid tissue (GALT), the intestinal branch of the body’s protection against pathogens (Spahn et al., 2006). MLNs serve as sites where DCs prime T cells for an immune response (Spahn et al., 2006). In our studies, decreased cell numbers were

observed in the MLNs with high dose notoginseng exposure. However, no changes in cell numbers were observed in the spleens and PBLNs, or with low doses of notoginseng. The change in the MLNs may be due to these immune tissues being exposed to the highest amounts of notoginseng constituents. Even after metabolism by gut flora, ginsenoside concentrations would be highest in the GALT areas following oral administration of notoginseng. This is because ginsenosides absorbed in the intestines have not yet been decreased by first pass metabolism. The decreased cell numbers could have been due to decreased ability of APC to induce T cell proliferation. Although we have not studied the effects of notoginseng on macrophages and DCs after continuous exposure to notoginseng, our previous studies demonstrated decreases in the activation and function of these cells after a single exposure to this herbal (Rhule et al., 2006b). However, T cell proliferation was not influenced by a single notoginseng treatment of DCs in these studies. Therefore, experiments could be designed to examine if continuous exposure to notoginseng could affect DCs ability to activate T cell proliferation. Additionally, it is also possible that notoginseng or its derivatives induced necrosis or apoptosis of cells in the MLNs leading to the observed decreased cell numbers.

The activation of B cells by soluble protein antigens such as ovalbumin requires the involvement of T helper (T_h) cells. The primary antibody type secreted after B cell activation is IgM. In order for other types of antibodies to be secreted, B cells have to undergo the process of class switching. This process cannot occur in B cells without signals from T_h cells such as CD40-CD40 ligand interactions. In our experiments, IgM levels were decreased at the 7-day treatment time point (3 days post immunization) with high doses of notoginseng. However, there were no changes in IgG levels at this time

point. This indicates that the effects of notoginseng may occur early in B cell activation before class switching from IgM to IgG occurs. Additionally, because the entire animal was exposed to notoginseng, it is unclear which immune cells or processes were directly affected by treatment. Notoginseng exposure could have altered a number of events including, antigen uptake, T cell interactions with B cells or their activation by APCs. There were no differences in IgM or IgG levels between PBS controls and notoginseng treated mice at day 17 of high dose and day 15 of low dose exposure. It should be noted at this time-point notoginseng was no longer being administered to the mice, as the 10 days treatment period was completed. It is therefore possible that the effects were “washed out” after a week of no treatment with notoginseng. Additional experiments focusing on the levels of antibody present in both low dose and high dose notoginseng mice over the entire treatment period are therefore warranted.

Previous studies indicated that notoginseng possesses adjuvant activity, increasing ova specific antibody levels in mice after treatment (Liou et al., 2005; Sun et al., 2007; Yang et al., 2007b). However, in our study ginseng reduced IgM levels at day 7 of high dose notoginseng exposure, with a trend towards decreased IgG production at the end of the experiment period in both high and low dose groups. The reason(s) for discrepancies between our studies could have been due to a number of factors including differences in the components of ginsenosides used in those studies compared to the sample of notoginseng used in our experiment.

Cytokines are important mediators of an immune response. TNF- α and IL-6 are among the first cytokines produced after immune stimulation. There was a reduction in TNF- α production in spleen cells stimulated *ex vivo* with LPS in high dose notoginseng

exposure, with an overall trend towards decreased cytokine production with both low and high dose notoginseng treatment. These decreases in TNF- α and IL-6 are consistent with our previous findings of reduced production of LPS induced pro-inflammatory cytokines with notoginseng treatment (Rhule et al., 2006b; Smolinski and Pestka, 2003a)(submitted article). In our previous studies, the effects of notoginseng on macrophages and DCs were examined in RAW2467 cells and bone marrow derived dendritic cells, respectively. As there are other cells present in the spleen that produce TNF- α and IL-6 in response to LPS stimulation, it is possible that these cells are not as sensitive to notoginseng treatment. Nevertheless, this possibility needs to be examined.

There were no changes in the cell surface molecule expression for any of the molecules tested. As these molecules are classic lineage markers for identification of different immune cell types, this suggests that none of the populations examined were being altered by notoginseng treatment. Overall it gives a good indication that low dose notoginseng treatment does not alter proliferation or increase apoptosis in the spleen, BPLN or MLN.

Although there have been a number of toxicological studies in ginseng species, studies on its immunotoxicological effects were not evident. In our study, we extended the previous findings on the high LD₅₀ associated with the *Panax ginseng* species, to *Panax notoginseng*. Our study also established for the first time that there are no immunotoxicological effects observed in mice treated with low dose notoginseng.

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CHAPTER 6

Panax Notoginseng reduces acetylated-LDL uptake by BMDCs

Unpublished data

Abstract

Panax notoginseng has been purported to decrease the effects of conditions such as diabetes mellitus, hypertension, hyperlipidemia and inflammatory diseases in a number of studies. These abnormalities are all known risk factors for atherosclerosis. Uptake of modified LDL by immune cells plays a major role in the early stages of atherosclerosis. In this regard, the effects of notoginseng on acetylated LDL (ac-LDL) uptake were examined. Bone marrow derived dendritic cells (BMDCs) were treated with notoginseng and exposed to FITC-conjugated ac-LDL. Notoginseng decreased ac-LDL uptake by immature BMDC as measured by flow cytometry. Furthermore, following stimulation with the inflammatory cytokine TNF- α , notoginseng effectively attenuated ac-LDL uptake by BMDCs. Expression of the accessory molecule CD40 was inhibited by notoginseng on TNF- α treated BMDCs. These studies suggest that notoginseng has the potential to alter uptake of modified LDL by antigen presenting cells.

Introduction

Inflammatory and immunologic mechanisms contribute to the initiation and progression of atherosclerotic lesions (Kinlay and Egidio, 2006). The earliest identifiable atherosclerotic lesion is characterized by immune cell infiltration and lipid accumulation (Hasham and Pillarisetti, 2006). These immune cells include activated macrophages, dendritic cells and T lymphocytes (Link and Bohm, 2002) (Ohashi et al., 2004).

There are a number of antigens that are thought to be responsible for cellular immune reactions in atherogenesis. Modified antigens such as oxidized low density lipoprotein (ox-LDL) are among the major endogenous activators of the immune system (Link and

Bohm, 2002). *In vitro* studies have demonstrated that elevated levels of modified LDLs including ox-LDL lead to activation and maturation of antigen presenting cells (APCs) such as dendritic cells (DCs) (Link and Bohm, 2002; Lord and Bobryshev, 2002). Activation of DCs by ox-LDL induces classic accessory/costimulatory molecule expression including CD40, CD86 and MHC II (Cao et al., 2003). This initiates increased T cell activation and proliferation, representing a chronic inflammatory response. This process involves T lymphocytes recognizing ox-LDL, resulting in an autoimmune response against cells bearing that antigen (Stemme et al., 1995).

The dietary supplement *Panax notoginseng* has been purported to have immunomodulatory as well as cardiovascular properties. Previous studies in our lab established that notoginseng reduced inflammatory mediatory production by both DCs and macrophages *in vitro* (Rhule et al., 2006b)(unpublished data). In a study with Wistar male adult rats on a fat-enriched diet, treatment with notoginseng decreased the total cholesterol and triglycerides present in the blood (Cicero et al., 2003). Additionally, notoginseng has also been demonstrated to reduce high blood pressure (Lei and Chiou, 1986). To date, there have been no studies examining the effects of notoginseng on modified-LDL uptake and atherosclerotic plaque formation. As both of these events have been demonstrated to have immune and cardiovascular components, we investigated the effects of notoginseng on ac-LDL uptake by bone marrow derived dendritic cells (BMDCs). BMDCs were treated with notoginseng and exposed to FITC-conjugated ac-LDL, a modified lipoprotein that is similar to ox-LDL, but more stable. In some experiments, BMDCs were exposed to the pro-inflammatory cytokine TNF- α , in conjunction with ac-LDL to simulate a mild inflammatory condition. Following

notoginseng treatment, levels of ac-LDL uptake and expression of the activation molecule CD40 on BMDCs were characterized by flow cytometry. Our study demonstrates that notoginseng reduces the uptake of ac-LDL by BMDCs and provides information on potential uses of this herbal for the treatment of atherosclerosis.

Materials and Methods

Mice

Male and female C57BL/6 mice aged 4-8 weeks old were bred and maintained in the animal research facilities at the University of Montana. Mice were housed under specific pathogen-free conditions and maintained on 12-hour dark/light cycles. Standard laboratory food and water were provided ad libitum. Protocols for the use of animals were approved by the University of Montana Institutional Animal Care and Use Committee.

Cells

Bone marrow cells (BMCs) were collected from the femur and tibia of C57BL/6 mice for *in vitro* use and differentiated in BMDCs as previously described in Chapter 4. Briefly, BMCs were grown in cRPMI with 30 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF), (Leinco, St Louis, Missouri) at 1×10^6 cells per ml in T75 flasks. Media and growth factor were replaced on days 3 and 5. Cells were harvested after 7 days and DCs purified (>90%) using CD11c-APC antibodies and anti-APC Miltenyi magnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions.

Chemicals

Noto-GTM extracts were kindly supplied by Technical Sourcing International, Inc. (TSI, Missoula, MT). *Panax notoginseng* (Burk.) F.H. Chen ex C.Y. Wu & K.M. Feng extracts was acquired from the root of the plant using ethanol and standardized to contain Rb1 and Rg1 ginsenosides at 35 and 34% of the whole extract, respectively. The quantification of Rb1 and Rg1 in the notoginseng extract was determined by high-performance liquid chromatography analysis by TSI. Certification of analyses was approved by Xia Ronglong (QA manager, TSI). Levels of *Escherichia coli* (E. coli) or *Salmonella enterica* were undetectable in the notoginseng preparation (unpublished data). The extract was dissolved in complete media (see below) and subsequently sterile-filtered through a 0.22 µm Millipore membrane. Acetylated FITC-conjugated LDL and TNF-α were obtained from Molecular Probes (Eugene, OR) and Peprotech Inc (Rock Hill, NJ), respectively.

Cell Activation and Treatment

BMDCs (1 X 10⁶ cells per well) were treated with 0 or 200 µg/ml of notoginseng and/or stimulated with TNF-α (10ng/ml) for 24 hrs at 37 °C and 5% CO₂ in 6-well plates. At 22 hrs of treatment, cell were incubated with 1 µg/ml ac-LDL for the last 2 hrs. Cells were harvested for FACS analyses. The viability of BMDCs used in all experiments was greater than 90% as determined by Trypan blue exclusion.

Flow Cytometry

Accessory molecule expression and fluorescent molecule uptake by BMDCs were performed by fluorescent activated cell sorting (FACS) analysis as previously described (Rhule et al., 2006b; Shepherd et al., 2001). Briefly, BMDCs that were previously incubated with FITC-conjugated ac-LDL were harvested and washed with PAB (1% bovine serum albumin and 0.1% sodium azide in PBS). Cells were blocked with 30 μ g of purified rat and/or hamster IgG (Jackson ImmunoResearch, West Grove, PA) for 10 minutes to eliminate non-specific staining. Fluochrome-conjugated monoclonal antibodies were used to stain cells for an additional 10 minutes. The antibodies used in these experiments were CD40-PE, CD11c-APC and their corresponding isotype controls (BDPharmingen, San Diego, CA). One hundred thousand viable cells per treatment (as determined by light scatter profiles and propidium iodide staining) were analyzed using a BD FACSAria flow cytometer and FACSDiva software (BD Biosciences, San Jose, CA).

Statistics

All statistical analyses were performed using GraphPad Prism 4.0a for the Macintosh (GraphPad Software, San Diego, CA). Data represents the mean \pm SEM of 3 samples for 3 independent experiments unless otherwise stated. Differences between treatment groups were analyzed by Student's t-test. Data sets with multiple comparisons were evaluated by one-way analysis of variance (ANOVA) with Dunnett's test. Values of $p < 0.05$ were determined to be significant.

Results

Notoginseng reduces ac-LDL uptake by BMDCs

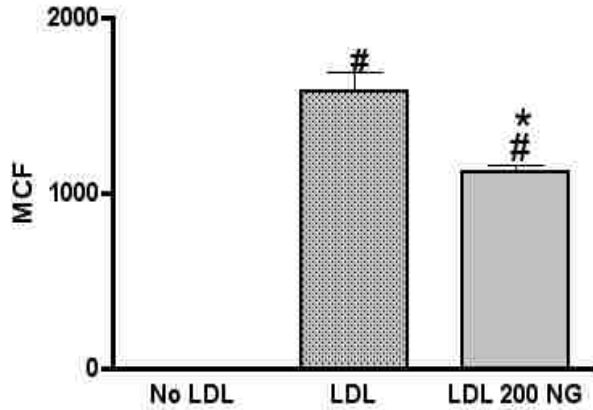
DCs are specialized innate immune cells with the ability to phagocytose antigens (Niedergang and Chavrier, 2005). Transformed-LDL has been recognized as one of the primary antigens present in the development of atherosclerotic lesions. In this regard, DCs were treated with 200 $\mu\text{g}/\text{ml}$ notoginseng and exposed to FITC-conjugated ac-LDL. There was a significant increase in the fluorescence intensity of BMDCs exposed to ac-LDL (Fig 6-1). Notoginseng reduced the uptake of ac-LDL by 30% when compared to untreated controls (Fig 6-1A). In some experiments, cells were exposed to TNF- α as this cytokine is associated with inflammatory responses within atherosclerotic plaques. Notoginseng decreased uptake of ac-LDL by TNF- α -treated BMDCs (Fig 6-1B).

CD40 expression on TNF- α -treated BMDCs is decreased by notoginseng

CD40 is a cell surface protein that is constitutively expressed on DCs and other APCs and is upregulated during inflammation (Lutgens et al., 2007). The interaction of CD40 and its ligand plays a significant role in the development and progression of atherosclerosis (Lutgens et al., 2007). Therefore, we examined the effects of notoginseng on the expression of CD40 on TNF- α -treated BMDCs after exposure to ac-LDL. CD40 expression was decreased on the notoginseng-treated BMDCs exposed to ac-LDL when compared to TNF- α -treated ac-LDL controls (Fig 6-2).

Figure 6-1.

A.



B.

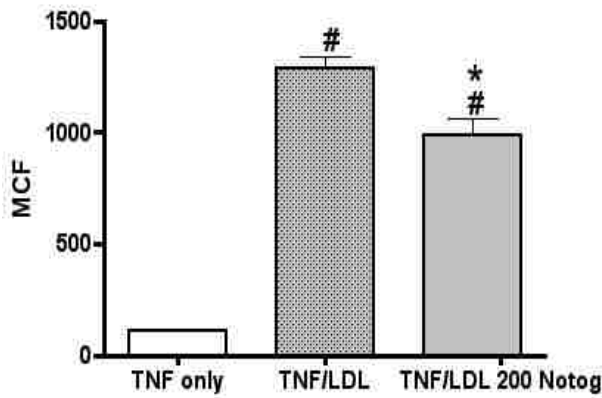


Figure 6-1. Notoginseng impairs ac-LDL uptake. BMDCs were treated with 200 $\mu\text{g/ml}$ notoginseng and/or stimulated with $\text{TNF-}\alpha$ for 24 hrs. Cells were exposed to FITC-conjugated ac-LDL 2 hrs prior to harvesting. Uptake of (A) ac-LDL and (B) ac-LDL by $\text{TNF-}\alpha$ -treated BMDCs was assessed by flow cytometry. Data represents mean \pm SEM of three samples. # indicates significant differences between LDL-exposed and unexposed cells; * indicates significant differences between LDL-exposed control- and notoginseng-treated samples ($p < 0.05$). Data are representative of three independent experiments.

Figure 6-2.

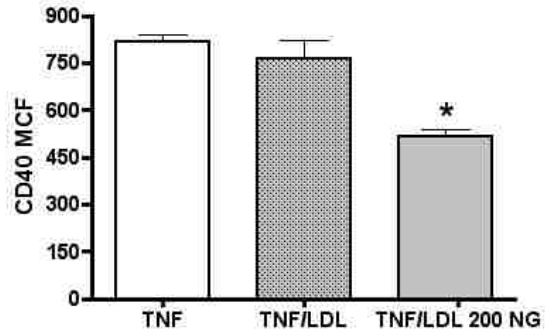


Figure 6-2. CD40 expression is reduced by notoginseng on TNF- α -treated BMDCs exposed to ac-LDL. BMDCs were treated with notoginseng and TNF- α for 24 hrs and then exposed to FITC-conjugated ac-LDL for an additional 2 hrs. The expression of the costimulatory molecule CD40 was assessed by flow cytometry. Data represents mean \pm SEM of three samples. # indicate significant differences between LDL-exposed and unexposed cells; * indicates significant differences between the LDL-exposed control- and notoginseng-treated samples ($p < 0.05$). Data are representative of three independent experiments.

Discussion and conclusion

Development of atherosclerotic plaques involves complex interactions between the endothelium, inflammatory cytokines, and numerous blood cells and components (Libby, 2004). Notoginseng has been shown to decrease inflammatory mediator production by a variety of immune cells. In this study, we investigated the effects of notoginseng on ac-LDL uptake by BMDCs. Using two models of exposure, notoginseng reduced ac-LDL uptake under non-inflammatory and mild inflammatory conditions. Exposing BMDCs to ac-LDL in the absence of TNF- α models the effects of notoginseng on antigen uptake.

However, there is considerable evidence that cytokines such as IL-1, IL-6, and TNF- α are involved in the formation of atherosclerotic plaques (Libby, 2004). In addition expression of cell surface molecules such as CD40 by cells in the plaque region fuels the inflammatory process by further leukocyte recruitment (Libby, 2004). Exposure of TNF- α -treated BMDCs to ac-LDL allows examination of the effects of notoginseng on antigen uptake during inflammatory conditions that would be expected in an atherosclerotic plaque. In our experiments, uptake of ac-LDL was reduced in notoginseng-treated BMDCs. Furthermore, CD40 expression was reduced on the notoginseng-treated BMDCs. This might lead to decreased activation of other immune cells within the plaque. However, this possibility remains to be examined.

Because notoginseng effectively reduced the uptake of ac-LDL by BMDCs, its potential to alter atherosclerotic plaque formation in ApoE/LDL receptor double knockout mice was evaluated (data not shown). These mutant mice are prone to develop atherosclerotic plaques (Bunderson et al., 2004). In these experiments ApoE^{-/-}/LDLr^{-/-} mice were orally dosed with 2.5 mg/ml of notoginseng in their drinking water over a ten-

week period. Unfortunately, plaque formation at the harvest (ten weeks) was not substantial enough to draw a conclusion between control-and notoginseng-treated mice. Therefore, these studies are presently being expanded to a time point of twenty weeks to facilitate more pronounced plaque formation.

Collectively, our data demonstrate that treatment of dendritic cells with notoginseng reduces their ability to phagocytose modified LDL. Furthermore, expression of the co-stimulatory molecule, CD40, was decreased by notoginseng on ac-LDL-treated BMDCs. Our results demonstrate that notoginseng may have the potential to decrease phagocytic activity of DCs in atherosclerotic plaque formation and provide provocative information on the use of this herbal for the potential prevention or treatment of cardiovascular disease.

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CHAPTER 7

Summary

In 1991 the NIH launched its office of Complementary and Alternative Medicine (now the National Center for Complementary and Alternative Medicine) in response to the public's huge interest in non-conventional medicine (<http://nccam.nih.gov/>, ; Kinsel and Straus, 2003). One of the largest subsidiaries of the alternative medicine group is dietary supplements, primarily the herbal products division (<http://nccam.nih.gov/>). Of these herbals, ginseng was the second most widely used by the U.S. population in 2002. Ginseng is purported to have numerous pharmaceutical effects including immunomodulation, lowering blood sugar and reducing hypertension (Carabin et al., 2000; Kitts and Hu, 2000; Lei and Chiou, 1986). However, the safety and efficacy of this herbal product has not been extensively examined. To address aspects of this deficiency we examined the immunomodulatory effects of *Panax notoginseng* on the fate and function of APCs. We hypothesized that notoginseng alters APC fate and function by decreasing activation molecule expression and inflammatory mediator production by macrophages and DCs. Furthermore, we believe that these effects may be exploited to treat immune-related diseases such as atherosclerosis and rheumatoid arthritis. As such, we characterized the immunomodulatory effects of notoginseng on APCs, the incidence of toxicity for notoginseng and eventually extended our studies to examine the effects of notoginseng on LDL uptake and atherosclerotic plaque formation in a mouse model.

In our study we examined the effects of notoginseng on APCs using the murine macrophage cell line RAW264.7, the dendritic cell line DC2.4 and primary BMDCs. As cytokines are important mediators of the inflammatory response, changes in the production of these proteins were initially assessed (Andreakos et al., 2004b). Notoginseng reduced the LPS induced production of the pro-inflammatory cytokines TNF- α and IL-6 in RAW264.7, DC2.4 and BMDCs. The use of BMDCs also afforded us the opportunity to test IL-1 β and IL-12 levels which were not produced in detectable amounts in the immortalized cell lines. The production of these cytokines was also inhibited by notoginseng.

Accessory molecule expression is integral for APCs to perform their primary function of activating T cells (van Kooten and Banchereau, 2000). In our experiments, expression of the key costimulatory molecules CD40 and CD86 was decreased on RAW264.7 and DC2.4 cells stimulated with LPS. However, CD86 proved not to be as sensitive to notoginseng treatment and required pretreatment of BMDCs to significantly affect its expression. In addition, MHC II expression was also not altered by concurrent treatment of BMDCs with notoginseng and LPS stimulation, but was decreased with pretreatment.

Inflammatory mediator production can be induced in immune cells via activation of toll-like receptors (TLRs) including TLR3, TLR4 and TLR9. In our studies, notoginseng attenuated TNF- α production and CD40 expression induced by TLR3, 4 and 9 activation in DC2.4 cells. Alternatively, CD86 expression was reduced by notoginseng when DCs were activated with ligands to TLR 3 and 4, but not TLR 9. Also, IL-6 production by notoginseng-treated cells stimulated with LPS and CpG was reduced when compared to controls. TLR 3 ligand-stimulated DCs were unaffected by notoginseng treatment. While

we do not fully understand the differential effects notoginseng on APCs, it is clear that DC responses to TLR ligands are affected by this herbal extract.

Inflammatory enzymes mediate immune responses by the synthesis of proteins or molecules capable of modulating the immune system. The mRNA expression of COX-2 was inhibited by notoginseng in RAW264.7 cells. Previous studies in our lab also established that the activity of COX-1/COX-2 was inhibited by notoginseng (Seaver and Smith, 2004). Future experiments should be carried out to examine if notoginseng might affect prostaglandin levels in DCs.

Activation of the transcription molecule NF κ B results in inflammatory mediator production by APCs. Notoginseng reduced the LPS-induced nuclear levels and activity of the p65 component of NF κ B in BMDCs. Future studies should examine whether other transcription factors such as Rel B and c Rel components of NF κ B, as well as non-NF κ B mediated pathways such as AP-1 are also affected by notoginseng.

A primary function of APCs is to phagocytose antigens for presentation to T cells. In this regard, we examined whether notoginseng affected antigen uptake by DCs. Notoginseng reduced ovalbumin antigen uptake by BMDCs. As antigen uptake as well as inflammatory mediator functions were impaired by notoginseng, we designed studies to examine whether notoginseng could alter the ultimate fate of DCs, the activation of antigen-specific T cells. Our studies demonstrated that notoginseng did not affect DC activation of antigen-specific T cells *in vitro* or *in vivo*.

Therefore, notoginseng reduced the production of inflammatory mediators and innate responsiveness by APCs, but did not affect the initiation of T cell-mediated adaptive

immunity. The long-term implications of our results suggest that notoginseng might be a natural product capable of treating various inflammatory diseases.

Based on these results, we subsequently evaluated if notoginseng generated any associated general toxicities or immunotoxic effects. Studies on the effects of high dose and low dose notoginseng treatment in mice showed limited toxicities following oral consumption of notoginseng. Moreover, these toxicities were only observed at the high dose of notoginseng tested.

Finally, we evaluated the effects of notoginseng on atherosclerosis, an important disease contributing to morbidity and mortality in developed countries (Keaney, 2000). Initial studies in our lab demonstrated that treatment of BMDCs with notoginseng resulted in decreased uptake of acetylated-LDL (ac-LDL). Additional experiments have been implemented to determine whether notoginseng decreases plaque formation in ApoE/LDL receptor, double knockout mice, which are prone to develop atherosclerotic plaques. Future studies in our lab will characterize the effects of notoginseng on plaque formation in these mice. The effects of notoginseng on the concentrations of inflammatory mediators in the serum of treated mice will also be analyzed.

In conclusion, notoginseng decreased the innate and inflammatory functions of APCs without altering their fundamental fate of activating antigen-specific T cell activation. Our studies demonstrate a low incidence of immunotoxicity associated with notoginseng consumption in mice. Taken together, our research highlights the potential usefulness of notoginseng in the treatment of inflammatory diseases. Future studies should characterize the specific mechanisms of action of notoginseng, and how this popular medicinal herbal can be exploited to treat diseases.

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