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Youssef Aachoui, Richard W. Fitch and Swapan K. Ghosh. Molecular and signatures of phytol-derived immunostimulants in the context of chemokine-cytokine microenvironment and enhanced immune response. In review

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3. Awarded the Jacob & Lydia Collicott Memorial Scholarship, Indiana state university, IN, USA 2011

Novel Phytol-Derived Immunostimulants (PHIS-01) for Enhancement of Vaccine Efficacy: A Comparative Study

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A dissertation

Presented to

The College of Graduate and Professional Studies

Department of Biology

Indiana State University

Terre Haute, Indiana

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

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by

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August 2011

Keywords: Vaccine, Adjuvants, Phytol derivatives, Diterpenoid

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ABSTRACT

Adjuvants are used widely in vaccine formulations. However for humans, choices are very limited. Since they are selected empirically, it is not expected that any two adjuvants would influence immune mechanisms the same way. However they all influence host microenvironment, antigen presentation, and retention of immunological memory. This study focuses on new terpenoid adjuvants based on phytol derivatives. We previously observed that phytol and one of its derivatives PHIS-01 (a phytol-based immunostimulant, phytanol) are excellent adjuvants. To gain an understanding of the structural features important for adjuvanticity, we further studied compounds derived from a diterpene Phytol. We designed two new phytol derivatives, PHIS-02 and PHIS-03 (aminated and mannosylated compounds respectively). In this study we investigated their relative safety and efficacy compared to PHIS-01 (phytanol) and other commonly used adjuvants that include alum, Freunds' adjuvants and SIS (extra-cellular matrix). In addition, we examined how changes at the polar terminus affect adjuvanticity of PHIS-01, PHIS-02, PHIS-03 in term of host microenvironment and safety profile. Using these adjuvants as emulsions with different soluble protein antigens, ovalbumin and a hapten-protein conjugate phthalate-KLH, we evaluated in both autoimmune resistant and susceptible murine models. The following immunological parameters were studied: 1) effects on antibody responses in terms of titers, specificities and isotypic profiles; 2) effects on T-helper cells, cytokines, and chemokines milieu; 3) involvements of apoptotic and/or necrotic activity and inflammasome pathways as their primary modes of action. Our results indicate that: 1)

modified phytol-derived adjuvants significantly augment antibody response of isotypes IgG1 and IgG2a, promote effective T cell proliferation and exhibit no adverse autoimmune anti-DNA response in either autoimmune or non autoimmune mice. 2) Phytol derivatives function by activation of antigen-presenting cells involving apoptotic/necrotic effects on target cells. 3) Phytol derivatives improve vaccine immunogenicity by promoting regulated and nonpathogenic inflammatory changes in the immediate microenvironments, as characterized by mobilization of chemo tactic factors (MCP-1, KC, MIP-1, LIX, lymphotactin, eotaxin), growth factors (MCSF, GCSF, GM-CSF), and cytokines that mobilize innate and adaptive immunity and lead to T helper polarization and a magnified antibody response 4) PHIS -01, compared to PHIS-03 and alum , is a better activator of genes in the inflammasome pathways. In conclusion, our findings also clearly highlight the importance of bonds and functional moieties in shaping the adjuvanticity of phytol derivatives. Hydrogenation of phytol generates PHIS-01 which is a very safe and superior adjuvant in terms of the quality and magnitude of the overall immune response evoked. However, modification of its polar terminus of PHIS-01 with a hydrophilic mannose moiety (PHIS-03) profoundly changes the cytokine/chemokine milieu and favors T-helper type 2 rather than the T-helper type1 induced by PHIS-01.

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ACKNOWLEDGMENTS

I am greatly honored and humbled to have had the opportunity to learn under the guidance of Dr. Swapan K. Ghosh. Dr. Ghosh provided me with an environment rich in learning and research accompanied with support and patience that allowed me to flourish. For this I am sincerely thankful and I hope for his continued guidance and colleagueship throughout the years. I would also like to express my gratitude to my dissertation committee members Dr. Gary Stuart, Dr. James Hughes, Dr. Allan Albig, and Dr. Richard Kjonaas. Their help, teaching, and scientific input have greatly improved my work and even though the constant revisions were frustrating at times I respect their endurance and dedication in helping me succeed.

Furthermore, I would like to thank my past and current peers including, Dr. Nisreen Al-shaibi, Dr. Hongtao Li, Roshni Roychowdry, Deepak Vijaya Kumar, Arun Seethram, and Marissa Kordy for they personal and professional help during my studies. It was a great pleasure to learn and do research around such as supportive and knowledgeable group of people. I would also personally like to thank Mrs. Rita Ghosh for I am a better teacher, and person having known and worked under her guidance. I would also like to express my deepest gratitude to Mrs. Tracy McDaniel, Ms. Sunshine Mack, Mr. El-hussein Chaqra, and Mrs. Maria Chaqra for their consistent help on administrative as well as personal issues has made my graduate experience a successful one. I would also like to thank Indiana Academy of Science, ISU Student Research Fund, Graduate School of Indiana State University, and Department of Biology without whom funding for this research would not have been possible.

Finally, last but most certainly not least, I would like to thank my family. I am very thankful to have a very loving, supportive, and caring family whose encouragement helped enlighten my path to finish my Ph.D. study in the U.S. It was difficult being so far away from my family and I would never have made it as far as I did without my sister Samira Aachoui whose amazing care and support helped make my graduate studies possible and enjoyable. I am indebted to you. Thank you all so very much in helping me achieve my career goals.

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CHAPTER 1

INTRODUCTION

Vaccine efficacy depends largely on two variables, the specific antigen(s) used, and the choice of adjuvants. It is the inclusion of the latter in a vaccine formulation that significantly improves the quality and magnitude of the specific immune response. Adjuvants are a diverse group of chemical compounds, and vary widely in their ability to influence the immune response. Due to lack of a clear understanding of their function, they are often regarded as "immunologists dirty tricks" [1]. There has recently been increasing interest in unraveling the mode of actions of adjuvants currently in use. The picture that emerged from adjuvant studies indicates that no two adjuvants work in a similar fashion, although the ultimate outcome for inclusion of an adjuvant is augmented vaccine efficacy, or in other words increased immunogenicity. Thus, the underlying principle in adjuvant selection is usually governed by consideration of their ability to influence the host's microenvironment, antigen presentation, and retention of immunogens. The latter is particularly important for sustaining immunological memory.

Historically, the field of vaccine research that spans over several centuries and has grown from simple use of cross-reactive pathogens to attenuated pathogens, and then to designer vaccines made of proteins, nucleic acids, or carbohydrate-protein conjugates. This transition has helped to make safer vaccines; however, the immune response to attenuated or

designer vaccines is less robust than the one with live pathogens. The problem with using live pathogens or offending agents is that they may overwhelm the immune system by their proliferative rates, toxicity, and the ability to evade immune surveillances. This necessitated the inclusion of substances that can non-specifically boost the effectiveness and immunogenicity of attenuated pathogens and designer vaccines by modulation of micro-environment and other undefined effects.

History of Vaccine Adjuvants

The concept of vaccines originated with the discovery of cow-pox vaccine by Ed Jenner in 1796. Subsequently, Louis Pasteur discovered and popularized the use of attenuated vaccine that helped to reduce or eliminate many infectious diseases. In the twentieth century, there have been many more successful vaccines including those that helped lower the incidence of polio, measles, mumps, and diphtheria. The fight is still not over since malaria and HIV are still playing havoc all over the world. Additionally, the offending agents are not always pathogens; the hazards may come from toxins, cancers, pollens, and many soluble and insoluble particulates. Even in these areas there are ongoing efforts to develop vaccines. Vaccines may be even useful in combating addictive compounds such as cocaine. The list and nature of offending agents grow larger from pathogens to non pathogens, and so grows the need for vaccines and adjuvants. The term adjuvant is derived from the Latin word *adjuvare*, which means to aid or to help. They are substances that nonspecifically stimulate the immune response to specific antigens. The concept of adjuvants arose in the 1925 from observations of Ramon *et al;* who noted that horses developing an abscess at the inoculation site of diphtheria toxin, generated higher titer of specific antibody[2]. They subsequently observed that an abscess generated by the injection of unrelated substances (agar, tapioca, lecithin, starch oil,

saponin or even bread crumbs), along with the diphtheria toxoid, increased the immune response against the toxoid [3]. One year later, Glenny demonstrated the adjuvant activity of aluminum compounds (Alum, i. e. , aluminum hyroxide) utilizing an alum-precipitated diphtheria toxoid [4]. In 1930, Freund developed the gold standard adjuvant that consists of a water-in-mineral oil emulsion containing killed mycobacteria, known as Complete Freund's Adjuvant (CFA)[5]. However due to its high toxicity, CFA cannot be used in human vaccines. Incomplete Freund's Adjuvant (IFA), which is the water-in-oil emulsion without added mycobacteria, is less toxic, has been used in some human vaccine formulations[6].

Although alum is currently the standard adjuvant in human vaccines, it suffers from many drawbacks, for example, alum cannot induce a T helper type 1 (Th1) cell- mediated immune response to fight certain viruses, bacteria and parasites [7, 8]. Alum or aluminum also has been linked to dementia, a loss of brain function that occurs with certain diseases [9]. In the past decade, significant efforts have gone into developing new vaccine adjuvants with good safety records and capable of activating both humoral and cell mediated response. In 1997, MF59, composed mainly of squalene, was the second adjuvant to be licensed for use in human vaccines [10]. Clinical studies show that MF59 is highly immunogenic allowing reduction of the dose of antigen used in vaccine formulations. This ability is of great interest, since most antigens are new recombinant peptides that are poorly immunogenic and available in limited quantities. Adjuvant MF59 also has been shown to be safe, however some of its components such as squalene have been found to be arthrithogenic in rodents [11-13]. These issues have raised questions about its safety and may restrict its use in vaccines.

Currently, many other adjuvants have been described with variable safety and immunostimulation records, however, despite all these efforts, the aluminum salt/gel-based

(alum) adjuvants remain the only standard versatile adjuvant licensed for human use in USA. The primary objective of our study has been to develop much safer and more broadly applicable adjuvants based on natural compounds such as terpenoids.

Adjuvant Classification and Possible Role

Vaccine adjuvants i.e., immune-potentiators or immune-modulators, have been consistently used to help mobilize cells of the immune system, and promote cross-talks between the innate and acquired immunity. Advantages of adjuvants include:

- Enhancement of immunogenicity of weak antigens and reduction of the antigen dosage required to engender a productive immune response
- Facilitation of antigen uptake, transport, and presentation by APCs by:
    - Increasing cellular traffic to injection sites.
    - Modulating the cytokine and chemokine environment necessary for recruitment and maturation of antigen presenting cells.
    - Up-regulating MHC class II molecules and the co-stimulatory molecules such as CD80, CD86 and CD40 ligand necessary to activate adaptive immunity.
- Optimization of an effective immune response to specific antigens through either:
    - Enhancement of the humoral response to antigen by stimulating rapid and sustained elicitation of antibodies of specific Ig isotypes.
    - Promotion of cell-mediated responses by inducting cytotoxic lymphocyte (CTLs) or NK T cells.
    - Improvement of immunological memory by vaccines.

Adjuvants can be classified based on their source and physicochemical properties or their principal mechanisms of action [14]. Based on physicochemical priorities, Edelman [15]

classified adjuvants into three groups: a) Active immune-stimulants that enhance the immune response to the antigen by directly activating APCs through the receptors of innate immunity such as Toll-like receptors (TLRs) or NOD (nucleotide oligomerization domain)-like receptors (NLRs), b) Carrier adjuvants, which are immunogenic proteins that mobilize T-cell help, and c) Vehicle adjuvants, such as oil-emulsions or liposomes that serve as delivery systems to facilitate the interaction of the antigens with the important cells of the innate immune system. Currently, adjuvants are mostly described into the following categories: gel-based adjuvants, tensoactive agents, bacterial products, oil emulsions, particulate adjuvants, fusion proteins or lipopeptides[16].

Mechanisms of Action of Adjuvants

Adjuvants are often regarded as “immunologists’ dirty tricks”. They enhance immunogenicity of co-administered antigens; however despite many efforts, their modes of action remain unclear. The inability to clearly elucidate how adjuvants exert their effects is due to the complexity and often multi-factorial nature of the mechanisms involved. However, general immunological events seem to be required for adjuvant effects. First, adjuvants prolong the persistence of antigens at injection sites [5, 17]. Second, adjuvants mobilize and up-regulate the innate immune system through facilitation of antigen uptake, transport, and presentation to the acquired immunity system. Finally, adjuvants modulate cytokine/chemokine micro-environments, thereby promoting cross-talk between innate and acquired immunity[18]. Adjuvants influence innate-immunity cells by: 1) increasing the recruitment of antigen presenting cells such as neutrophils, monocytes, eosinophils, immature dendritic cells (iDCs), and macrophages to sites of injection, 2) promoting APCs ability to uptake antigen, 3) up-regulating MHC class II, B7-1 (CD80) and other co stimulatory

molecules expression on APCs, 3) up-regulating the expression of pro-inflammatory cytokines and chemotactic factors necessary for recruitment, maturation, and activation of both innate and adaptive immunity. The issue is how a nonspecific substance such as an adjuvant stimulates the non-specific arms of the immune system and ultimately leads to specific immune response by the vaccine. Two working models have been approached and been shown to control the initiation and progression of the immune response. These models are the stranger model of Janeway [1] and the danger model of Matzinger [19].

The Stranger Model

Adjuvants facilitate cross-talk between innate immunity and acquired immunity essentially by influencing host microenvironment with optimum mobilization and deployment of APCs such as neutrophils, eosinophils, DCs, and macrophages. APCs present in all tissues pick up antigens from local environments. However, these APCs are not in an immuno-stimulatory state, which makes them unable to activate T cells. In Janway's Stranger hypothesis, APCs are equipped with pattern-recognition receptors (PRRs) that recognize unique features of microbial molecules (pathogen-associated molecular patterns, PAMPs)[1]. Toll-like receptors (TLRs) represent an important family of PRRs that recognize PAMP [20]. In the presence of PAMPs such as LPS, CPG or other TLR ligands used as adjuvants or from infection, immature DCs (iDCs) become activated mature DCs by uptake of antigens via receptor-mediated endocytosis or pinocytosis. The DCs migrate to secondary lymphoid organs, and present processed antigenic peptides to naïve T cells in the context of MHC molecules (Figure 1).

Activation of Toll-like Receptors (TLRs)

Toll-like receptors are evolutionarily conserved and homologues to those found in insects, plants and mammals [21]. TLRs were first described as developmental protein required for anti-fungal immune responses in the adult *Drosophila* fly [22]. TLRs are expressed on the surface of several immune cells such macrophages, DCs, B cells, and other cell types [23]. They consist of a type 1 trans-membrane protein containing leucine rich (LR) extracellular domain for recognition and a Toll-IL1-R cytoplasmic tail that initiates intracellular signaling events[24]. Engagement of TLRs by their ligands such as LPS or CPG induces the transcriptional activation of gene encoding chemokines, pro-inflammatory cytokines and co-stimulatory molecules. In turn, these genes control the activation of inflammatory cytokines, type1 interferon, and chemotatic factors. TLR consists of ten family members that differ by ligand specificity, cellular localization, and downstream signaling (Figure 2) [25]. Various TLR ligands trigger different types of innate immune responses. Based on the types of ligands detected, TLRs can be divided into several families [26]. TLR1, TLR2 and TLR6 recognize lipid type of ligands, while TLR7, TLR 8 and TLR9 recognize nucleic acids. Some TLRs recognize unrelated ligands, for instance, TLR4 recognizes LPS, heat-shock proteins, respiratory syncytial virus, and the plant product paclitaxel. TLRs also differ in their cellular localization [26]. While TLR1, TLR2, TLR4, TLR5, and TLR6 are all receptors located on the cell surface; other TLRs (TLRs 3, 7, 8) are located within the endosomes and mainly recognize ligands such as extracellular nucleic acids that require internalization via endosomes.

Regardless of the stimulus, most TLRs activate similar downstream signaling events via IL1-R cytoplasmic tail [27]. This signaling results in activation of NF-Kβ and MAP kinase, and culminates in the regulatory response. Upon stimulation, TLRs mediate an interaction

between TIR domain–containing cytosolic adapters, including myeloid-differentiation primary response protein-88 (MyD88), TIR domain–containing adapter protein (TIRAP), TIR domain–containing adapter–inducing IFN-β (TRIF), and the TRIF-related adapter molecule (TRAM). Activation of MyD88 initiates downstream signaling through Irak1, which in turn activates NF-kβ and MAPk pathways [28]. Other TLRs such as TLR3 are Myd88 independent; they function by inducing the phosphorylation of IRF3 and IRF7 (Figure2) [29].

Activation of NF-kβ pathway induces inflammatory response mediated by pro-inflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α, and IL-12), chemotactic factors (Rantes, MIP-1α, MIP-2β). Also, activation of IRF3 results in up-regulation of co-stimulatory molecules (CD40, CD80 and CD86) necessary to stimulate T cells [30].

Danger Model

The Stranger model provides insight into the way immune systems counter-attack non-self agents such as pathogens, but does not explain how or why a robust immune response is generated to modified self as in cancer and some autoimmune diseases. These exceptions led Matzinger in 1994 to propose the Danger hypothesis. Matzinger proposed that the immune system has evolved to respond to non-physiological cell death, damage, or stress rather than only to infectious agents [19]. According to the Danger model, dying cells release endogenous adjuvants simply called danger-associated molecular patterns (DAMPs) that have the ability to stimulate local APCs, and in turn activate the adaptive immunity (Figure 1)[31]. This has led to questions about whether all adjuvants function by promoting cell deaths which attract and activate the innate and acquired immunity. In other words, the action of vaccine adjuvants involves apoptotic/ necrotic events at injection sites that draw in various players of the immune system.

Apoptosis and necrosis are the main mechanisms of cell death. Apoptosis, also called programmed cell death, is characterized by condensation of the chromatin, changes in cell shape and size, and flipping of the phospholipid phosphatidylserine to the cell surface from the cytosolic side[32]. Necrosis, on the other hand, is sudden cell death due to injury, and characterized by plasma membrane rupture, and release of cytoplasmic constituents[33, 34]. Several researchers have reported important roles for both types of cell deaths during immunological process. Apoptotic cells are cleared from the circulation by phagocytic cells such as macrophages. Epitopes from phagocytized materials can be presented through MHC molecules to T cells[35]. As it has been shown, immature DCs acquire epitopes from engulfed materials and present them through MHC molecules to CD4+ and CD8+ cells and induce T helper and cytotoxic T cells. This phenomena of presentation of exogenous antigen through both MHC class I and class II molecules is known as cross-priming [36].

The ability of the innate immune system to respond to dying cells is largely due to the presence of receptors on immune cells that are able to recognize DAMPS. This interaction initiates a cascade of downstream events leading to activation of antigen presenting cells and subsequently adaptive immunity. DAMPs are normally sequestered inside the cells as cryptic epitopes of cellular proteins or other structures [31, 37]. They are released mostly during necrosis. In fact, necrotic cells are better able to activate the immune system than apoptotic cells. Sudden cellular injury during necrosis damages cellular membrane integrity which causes the release of DAMPs. In contrast, apoptotic cells maintain their membrane integrity, and prevent leakages of DAMPs outside the cells. However, when these apoptotic bodies are not rapidly cleared by phagocytes, dead cells undergo a secondary necrosis, eventually lose membrane integrity and release intracellular content to the extra cellular environment[38].

Several criteria have been established to qualify a molecule as a DAMP. Candidate DAMP molecules are distinct chemical entities, and their biological activity is not due to contamination with pathogen-associated molecules pattern (PAMPs). Based on these criteria, only a few molecules have been recognized as DAMPS. One way adjuvants function is by unleashing DAMPS and other cryptic domains of cellular elements [38].

Among DAMPs released in response to adjuvants activating the immune system are uric acid, high-mobility group protein B1 (HMGB1), and heat shock proteins (HSPs). Uric acid, or more precisely monosodium urate (MSU) microcrystal, can stimulate DCs in vivo and vitro [39, 40], and decreasing MSU through uricase was shown to abolish the antibody response to OVA following stimulation by alum [39]. Heat shock proteins were the first DAMPs to be identified. Their adjuvanticity is evident in their ability to deliver associated peptides to antigen presenting cells [41]. Furthermore HSPs stimulate and induce the maturation and migration of APCs to lymphoid organs [42]. HMGB1, an intracellular DNA binding protein also has been shown to act like an endogenous adjuvant by its ability to function as a pro-inflammatory cytokine [43, 44]. Additions of HMGB1 to a vaccine formulation caused an increase in antibody response to soluble antigens, and induced cellular protection to tumor challenge [45]. While all these molecules possess adjuvant activity, their mechanisms of action are quite different. HSP and HMGB1 being TLR ligands stimulate the innate systems by initiating downstream signaling through TLRs [46, 47], whereas uric acid has been shown to activate innate immunity through binding to NOD-like receptors and activation of inflammasomes [48].

"When a cell dies *in vivo*, the event does not go unnoticed" *Hajime Kono and Kenneth L. Rock* [38]

Effectively, once DAMPs are released, they interact with surface receptors such as TLR or NOD-like receptors, thereby stimulating pro-inflammatory cytokines, growth factors and chemokines such as IL-1, G-CSF, and MCP-1. These mediators act on surrounding tissues, especially vascular endothelium, causing them to become leaky and orchestrating the recruitment of neutrophils, monocytes and macrophage. These cells phagocytize dead cells, repair injured tissue, and activate adaptive immune cells [38, 49].

Activation of NOD-like Receptors (NLRs) Family and Inflammasome

The innate immune system utilizes besides TLRs, other PRRs such NOD-like receptor (NLR) family genes that promote and stimulate specific immune responses. In contrast to TLRs, NOD-like receptors sense and recognize PAMPs or DAMPs in the cytosol [50-52]. There are 22 NLR family genes in humans but many more in mouse; these genes are categorized into 3 sub-families, NOD-like receptors (NOD1-2, NLRC3, NLRC5, NLRX1, CIITA), IPAF (NLRC4, NAIP4-5), and NLRP (NLRP1-14)[53, 54]. These receptors consist of a nucleotide-binding and oligomerization (NACHT) domain, often flanked by a C-terminal 11 leucine-rich repeat (LRR) domain and an N-terminal caspase recruitment (CARD) or purine domain (Figure3). Upon activation of members of a NOD-like receptor family such as NLRP3, a complex is formed with an adaptor protein called ASC and procasapse1. The resulting complex is like a platform designated as the inflammasome (Figure 4) [55]. Several NLRs family members have been reported to form inflammasomes *in vitro*, however only a few NLRs family member have been explored for their physiological activities in vivo [53]. These inflammasome platforms are referred to as IPAF, AIM2 and NLRP3 inflammasomes: Among these, NLRP-3 is the best characterized inflammasome that is linked to adjuvanticity of alum [56].

NLRP3 (nucleotide-binding domain leucine-rich repeat containing family pyrin domain containing 3) is activated by a plethora of diverse molecules including viral DNA [57], pore-forming toxins [58], and endogenous adjuvants such as gout-associated uric acid crystals [48], extracellular ATP [58] or amlyoid beta [59]. The ability of NLRP3 or other NLR family genes to detect endogenous adjuvants further explains and corroborates the simplistic danger model based on self/non-self recognition proposed by Matzinger. Since most of the endogenous adjuvants are hidden (cryptic) inside the cells, they are considered non-self epitopes or antigens. These antigens can be released under metabolic stress or tissue injury due to necrotic effects caused by some adjuvants such as alum. Activation of NLRP3 leads to formation of an inflammasome platform that recruits casapse1[60]. Caspase-1 regulates the processing and secretion of highly potent pro-inflammatory cytokines IL-1β, IL-18, and IL-33 [61]. Upon cleavage of their pro-forms by caspase-1, these cytokines become active and mediate several effects critical for the inflammatory response. IL-1β produced mainly by monocytes and macrophages is a potent pro-arthritogenic cytokine activated in response to injury and infection [62]. Also IL-1β induces secretion of many pro-inflammatory cytokines, chemokines and promotes the expression of inflammatory mediators such as: adhesion molecules, endothelin-1, and inducible nitric oxide synthase in endothelial cells [63-65]. Conversely, IL-18 and IL-33 can effectively influence the type of adaptive immune response. Cytokine IL-18 induces INF- γ expression and promotes differentiation of T helper type 1 cells and a cell mediated response [66]. Conversely, cytokine IL-33 activates T helper 2 cells, which in turn stimulates the humoral immune response [67].

Mechanisms of activation of NLRP-3 are not yet completely understood but three models based on the initial ligands have been proposed and supported in literature (Figure 4)

(reviewed in reference [53]). In model 1, extracellular ATP stimulates the pyrogenic P2xt-ATP-gated ion channel [68], which in turn triggers the K+ efflux, and induces the recruitment of Pannexin-1 membrane pore channel [69]. This allows NLRP-3 agonists to gain access to the cystosol where they bind to NLRP3 [70]. The second model involves the activators that form crystalline or particulate structures such as uric acid, abstestos, or amyloid-β. Due to their physical characteristics, engulfment of these activators causes lysosomal rupture. NLRP3 senses and engages lysosomal contents such as cathepsin-B [71, 72]. Finally the last model argues that activation of a reactive oxygen species (ROS)-dependent pathway triggers NLRP-3 inflammasome activation [73-75]. Interestingly, most of the danger-associated molecules (DAMPs) or pathogen associated molecular patterns (PAMPs) cause the generation of reactive species (ROS) [73-77].

Besides regulating IL-β, IL-18, IL-33, inflammasomes are thought to play other important physiological activities. These activities are yet to be clearly delineated.

Disregulation of the inflammasome may be responsible for many inflammatory disorders such type II diabetes, gout, pyogenic arthritis, etc. Regulation of the inflammasome is mediated by several proteins, for instance: 1) CARD containing proteins such as caspase 12, which suppresses inflammasomes by preventing caspase1 recruitments [78]. 2) Anti-apoptotic proteins such as BCL-2, whish inhibit inflammasomes by suppressing NLRP-1-dependent casapase1 activation [79]. 3) NLRP12 that inhibits NF-Kβ pathways and suppresses IL-1β gene expression [80, 81].

Activation of Adaptive Immunity

Differential activation of APCs through PRRs plays an important in linking innate immunity to adaptive immunity. DAMPs and PAMPs can independently alert the immune

system to an infection or a danger signal and possibly even in a synergistic manner. This might be responsible for different profiles in activation and regulation of adaptive immune response. Thus, the efficacy of adjuvants does not only stop at increasing the immunogenicity of antigens included in vaccines, but also affects the quality and magnitude of the adaptive immune response mounted against it. Vaccines work by stimulating antibody responses, as well as cellular immune responses involving Th1 cells and cytotoxic T lymphocytes (CTLs). Such responses are required to control chronic infectious diseases associated with viruses and intracellular pathogens, and also for the development of therapeutic vaccines against cancer (2). DCs become the most efficient APCs as they orchestrate the differentiation of T-cells into Th1 or Th2 profiles (Figure 5) following an encounter with the invading pathogens or inflammatory stimuli delivered by adjuvants in the body [82]. Three different subsets of DCs have been identified, each with the potential to activate the adaptive immune system in a very distinctive way. Lymphoid DCs are effective in inducing a cell-mediated immune response via their ability to secrete IL-12 which primes Th1 cells. On the other hand, activation of myeloid DCs caused secretion of IL-4 to activate Th2 cells that subsequently activate B cells and lead to production of antibody [83]. Effects of adjuvants on adaptive immunity are manifested in type of activated T cell that is activated. For instance, the LPS as adjuvant engages theTLR4 receptor and leads preferentially to Th1 like response, while Pamcys3 through interaction with TLR2 activates a Th2 response [83, 84]. Cancer cells are considered as self-antigens, thus mounting an anti-cancer immune response means the breakdown of tolerance to self components, and the consequence may be an autoimmune disorder. Previous reports have shown that DCs can indirectly elevate this negative blockade. IL-6 produced by DCs promotes

induction of cell mediated response directed against life-threatening cancer cells by inhibiting the $CD4^{+}25^{+}$ T regulatory cells that clamp down on anti-self reactivity [85, 86].

Modulation of Cytokine and Chemokine Micro-environment by Vaccine Adjuvants

A productive immune response is defined by the generation of clonally expanded antigen-specific T and/or B cells. The generation of such responses requires two kinds of stimuli. Signal1 is provided by the presentation of antigens by anti-presenting cells (APCs) through MHC molecules to specific T-cell receptors on naïve T cells [87]. Signal 2 is delivered by the co-stimulatory molecules induced by cytokines released by the APCs. These signals contribute to the priming of T helper cells and their subsequent interactions with antigen-specific B cells and cytotoxic T cells (CTLs)[88]. The vaccine adjuvant system impacts the immune response inducing secretion of bio-response modifiers consisting of cytokines, chemokines, and growth factors at injection sites. Thus, these bio-response modifiers control a complex network of regulatory events, which contribute to the differentiation and cross talk between immune cells.

Following immunization, several chemokines (Figure 6) are rapidly induced, as the early response modifiers, and their levels return to "baseline" within a day. This rapid increase in chemokine expressions is aimed at increasing the traffic of antigen-presenting cells to site of injection [89, 90]; for instance, KC and LIX (both neutrophil chemotactic factors) and eotaxin (eosinophil chemotactic factor) are stimulated as early as 2 hours prompting migration of neutrophils, eosinophils and basophils [39]. MCP-2 (monocyte chemotactic factor) another chemokine shown to be secreted after immunization of several adjuvants such as alum and MF59, promotes recruitment of monocytes that can differentiate into DCs, and prime naïve T cells [91]. Other chemokines often induced after exposure to adjuvants include macrophage

chemotactic factors (MIP- related proteins), activated T cells, iDCs and NK cells chemotactic factors (TCA-3, lymphotactin) [90, 92, 93]. The majority of these pro-inflammatory chemokines are induced by IL-1ß or tumor necrosis factor-α (TNF-α)[89]. The latter are often induced in response to pathogens or danger signals. Other chemokines such as MIG, IP-10 are up-regulated by interferon-γ (IFN-γ) [94]. Chemokines control cell migration by binding to several different receptors on leukocytes (Tables 1) [95]. These receptors are differentially expressed by distinct leukocyte subsets, which govern not only an important component of the specificity of chemokine actions [96, 97], but provide a high degree of effectiveness and flexibility of the immune response *in vivo* [95].

Chemokines are involved in more than the control of cell trafficking. RANTES was shown to induce eosinophil and basophil degranulation, and the respiratory burst in eosinophils [98], and augment T cell proliferation [99]. Platelet factor 4 (PF4) inhibits megakaryopoiesis [100] and manifests bactericidal effects [101]. In addition, some chemokines such as LIX, eotaxin and growth factors are involved in hematopoiesis [102-105]. Furthermore, differential chemokine receptors expression plays a crucial role in the trafficking, tissue infiltration of different T cell subsets, and the generation and direction of Th1-type or Th2-type immune responses. These response are due to expression of different specific chemokine receptors on T helper cell subtypes[106]. Th1 cells have been shown to preferentially express the chemokine receptors CXCR3 and CCR5, while Th2 cells express CCR4, CCR8, and some CCR3 [106-108].

While most chemokines appear to control the innate immune system, cytokines are probably more active in orchestrating the adaptive immune system. The Th1/ Th2 paradigm plays a central role in response to various treatments (Figure 5). CD4+ T-cells have been

classified into two subsets (Th1 and Th2) according to cytokines they produce [109-111]. IFN-γ, IL-2, leukotriene A, GM-CSF are cytokines produced by Th1 subtypes. These cytokines stimulate strong cell-mediated CTL responses, delayed-type hypersensitivity (DTH) reactions, and induce Ig subclasses IgG2a and IgG2b. Th2 subtypes produce cytokines IL-3, -4, -5, -6, -10, and -13 that evoke a strong humoral or antibody-mediated immune response, with the induction of IgG1 and IgE antibodies. In addition, cytokines of both types involved in Th1 and Th2 function can be secreted by a third subtype called Th0 that is believed to give rise to the "polarized" Th1 and Th2 lineages. Cytokines released by the antigen-presenting cells (APCs) in response to different stimuli are important factors for determining which classes of T helper cells will emerge. The early production of IL-12 has been shown to prime Th1 responses, while the secretion of IL-10 biases towards Th2 T-cell responses. In addition, Th2 cytokines have been shown to inhibit Th1 differentiation. Other cytokines such as transforming growth factor-ß (TGF-ß) secreted by Th3/T-regulatory-1 T cell subsets are shown to inhibit any ongoing immune response possibly by down-regulating the antigen- presenting cells [112].

All the microenvironments described above are seen in immune-competent subjects. In order for a vaccine to be effective in autoimmune prone individuals, adjuvants should be ideally non-toxic and should not promote chronic inflammation.

Differential expression and interaction of chemokines and cytokines with target cells is highly involved in the onset and perpetuation of the autoimmune response and tissue damage in lupus. During renal diseases in lupus-prone mice, the infiltration of monocytes/macrophages, B1 cells and T cells into kidneys is controlled by elevated expression of chemokines such as, BLC, MCP, RANTES, MIP-1α. These chemokines have been shown to play a central role in progression and severity of renal disease. Following the early chemokine expression, the

progression of the diseases has been linked to up-regulation of cytokines such as TNF-α, IFN-γ, IFN-ß, interferon-γ inducible factor, and IL-1β. On the other hand, production of anti-inflammatory cytokines such as TGF-β, IL-10 is down regulated. An imbalance between Th1 and Th2 cytokines appear to be a hallmark for lupus. Analyses of sera from lupus patients showed increased levels of IL-4, IL-10, IL-12, and IL-18. This change in Th1 or Th2 cytokines affects the balance between immune-protection and/or immune-pathology. Therefore adjuvants can modulate systemic or local chemokine and cytokine profiles, and thereby aggravate or ameliorate autoimmune disease progression and/or pathogenesis.

Review of Clinically Approved Adjuvants

Despite a rich knowledge regarding the mechanisms of action employed by adjuvants and the immune system function, only a few adjuvants are approved for human use. This is partly because of unacceptable side-effects and toxicity associated with some adjuvants. The list of adjuvants approved for human use includes alum, MF59 and Adjuvant systems (ASs). Here we describe the effects of these adjuvants and other adjuvants used during this study:

ALUM

Alum containing adjuvants are the most widely used immune-potentiators in human vaccines [1]. Alum salts are inorganic water soluble compounds, from which two alum adjuvants are licensed for use in human, aluminum hydroxide ($Al(OH)_3$) and aluminum phosphate ($Al(PO_4)$). These compounds are simply known as alum. Alum contains electrostatic binding sites that allow antigens to be adsorbed and slowly released over time to stimulate an enhanced immune response [113]. Adsorption and the slow release of antigens are also thought to be important parameters for the efficacy of alum and also for reducing the severity of local and systemic inflammation. The latter is likely responsible for alum's good

safety record. Alum adjuvants induce a protective Th2 immunity characterized by IgG1 isotype antibodies [114]. However a major drawback of alum is its inability to induce much of a Th1 response needed to combat certain viruses, bacteria, and parasites[8]. Furthermore alum can induce an IgE antibody response, which may predispose susceptible individuals to allergic reactions.

Alum-containing adjuvants have been used routinely in vaccine formulation over 80 years, yet their mechanisms of action still remain unclear. Following intramuscular injection, alum rapidly induces innate immune cells to release chemokines and cytokines such KC, eaotaxin, MCP-1, and IL-8. These chemokines attract neutrophils, eosinophils, monocytes, and particularly the inflammatory Ly6c+CD11 b [39]. Alum also induces the release of uric acid from damaged surrounding tissues at the sites of injection [39]. Innate immune cells, especially monocytes, react to the uric acids and alum through activation of NLRP3 inflammasomes, uptake the antigen and process it into small peptides bound by MHC molecules [56]. As monocytes differentiate into DCs, they migrate to lymph nodes where they activate antigen-specific effector T cells. In the spleen, alum facilitates recruitment of $Gr1^{+}IL4^{+}$ eosinophils, and stimulates B cells [115, 116].

MF59

MF59 is an oil-in water emulsion that is a safe and effective adjuvant[10]. Although its use in human vaccines is restricted in the USA, MF59 is used worldwide in human vaccines, especially with flu vaccines in European countries [117]. MF59 contains mainly squalene, a natural triterpene found in shark liver oil which serves as precursor for cholesterol. MF59 enhances immune responses to a wide range of co-administered antigens. MF59 adjuvanticity is largely due to its ability to “jump start” the innate immune response. MF59 induces chemo-

attractants and cytokines (MCP-1, IL-1β) that are able to recruit monocytes and granulocytes to the sites of injection [91, 117]. MF59 also enhances and accelerates differentiation of monocytes into DCs and leads to up-regulation of CCR7, the DC homing receptor for draining lymph [117]. MF59 modulates the immune response without biasing toward Th1 or Th2. This is clearly demonstrated as DCs generated after MF59 stimulation were potent at inducing T cell proliferation and secretion of slightly more IFN-γ and slightly less IL-5. INF-γ and IL-5 induce Th1 and Th2 cells respectively [118].

MF59 is a well tolerated adjuvant; however, conflicting reports have raised concern that squalene may trigger the production of anti-self antibodies causing autoimmune disorders. In fact, soldiers suffering from symptoms of gulf war syndrome were found to have natural antibodies to squalene [119].

Freund's Adjuvants (for veterinary use)

Complete Freund's adjuvant (CFA) is possibly the gold standard for adjuvants. This is due to its high effectiveness in enhancing an immune response to co-administered microorganisms or harmless proteins. However, due to its high toxicity, its use restricted to veterinary medicine [120]. CFA is composed of a mixture of paraffin oil containing mannide mono-oleate as a surfactant, and heat-killed mycobacteria. CFA forms a viscous water-in-oil emulsion with suspensions of antigens, thereby prolonging antigen persistence at the sites of injection. Immunization with CFA induces high levels of circulating antigen-specific antibodies, strong T-lymphocyte responsiveness, and a delayed type hypersensitivity (DTH) - reaction [121]. The mycobacterium in CFA is a PAMP that targets the innate immune system through toll-like receptors. Exposure to CFA (or mycobacteria) induces a local inflammatory response, characterized by release of the chemo-attractants MCP-1 and IL-8 as well as the pro-

inflammatory cytokines TNF-α, IL-12, IL-6, IFN-γ that attract and promote DCs maturation [121, 122]. Mycobacterial components of CFA induce the production of monokines, in particular IL-12 and TNF-α. IL-12 induces NK cells to produce IFN-γ, which potentiates production of IL-12, and promotes Th1-type immune responses to CFA. Incomplete Freund's adjuvant (IFA) has the same components as CFA but without the mycobacteria. Both IFA and CFA act as adjuvants for the production of antibodies. However immunization with IFA unlike CFA is infective for induction of a cell-mediated response. Also, by lacking PAMPs, it fails to stimulate APCs, it can only favor development of a strong Th2-type response [121, 123, 124].

Other commercially available adjuvants that are considered for human trials contain a series of adjuvant systems called AS. ASs are a cocktail of adjuvants containing MPL A; the latter is a safe detoxified derivative of LPS and saponin purified from the bark of quillaja sponaria Molina. The ASs systems induce a strong humoral response and a long lasting cellular memory to hepatitis B surface antigen [125]. The mechanism of action of the AS adjuvant is thought to be mediated first by activation of TLR4, since MPL A is a TLR4 ligand, and secondly the release of endogenous adjuvants due to intrinsic lytic activity of saponin on local tissue at sites of injection [7].

Overall Objective

In order to make widely usable, safe, and effective adjuvants with defined algorithms, we focused on terpenoids. We assessed their adjuvanticity in the context of following biological effects:

1) Retention of antigen to promote a sustained immune response.
2) Activation and promotion of the interaction between innate and acquired immunity by changing cytokine and chemokine micro-environments.

3) Induction of non-pathological inflammatory responses.
4) Minimization of doses to overcome cumulative toxic effects
5) Induction of long-term immunological memory
6) Prevention of autoimmune responses
7) Activation of desired immune response involving all components of the immune system.

Rationale for Designing of Phytol Derivatives

Phytols (3, 7, 11, 15-tetramethyl-2-hexadecen-1-ol) are terpenoids. Terpenoids, also called isoprenoids, are organic molecules derived from five-carbon isoprenes. Isoprene units are assembled and modified in many ways to give birth to the 25,000 terpene structures reported so far [126]. As the largest class of natural products, terpenes have a variety of roles in nature that can be classified to 3 categories: function, defense, and communication.

Functional role: terpnes function in the biosynthetic pathways of many molecules used in organisms. For example; sterols are important components of the cell membrane, vitamin A (retinol) is used as a precursor for the synthesis of eye pigments responsible for vision, and vitamin E (tocopherol) is used as an antioxidant that prevents cell damage [126, 127]. Terpenes such as farnesyl or a geranyl-geranyl facilitate attachments of proteins important for cell signaling and cell organization to cell membranes by a process called prenylation, as seen for nuclear lamins [128, 129].

Role in Defense: terpenes function as toxins or repellents to other organisms. Drimane sesquiterpenes are widely utilized by plants, fungi, and certain marine organisms as potent antibacterial and antifungal compound [130]. Terpenoids can be toxic to insects, nematodes,

mollusks and fish. Bufotalin, a terpene which functions as heart stimulant, is produced by toads to prevent other animals from preying on them [126].

Role in communication: terpenoids can function as chemical messengers such as sterols, or steroids that function as hormones. Terpenes such as sesquiterpene (E)-b-farnesene serve as sex, aggregation, trail, and alarm pheromones [131, 132].

Our interest in terpenoids comes from their ability to enhance the immune system. Epidemiological studies have shown that intake of green vegetables rich in isoprenes enhance resistance to infection and improve immunity to cancer [133]. Furthermore isoprenoids suppress the growth of tumor cells *in vivo* and *in vitro* [133, 134]. Terpenoids such as squalene, are used in vaccine formulations for human and have been shown to provide a protective immunity against external threats[118]. However some of these compounds can be toxic and have arthritogenic activity [135-138]. Prior studies have linked phytol to Refsum disease, an autosomal recessive disorder that results from the accumulation of unmetabolizable phytanic acid in tissues[137]. Also pristane, a naturally occurring diterpenoid alkane, has proven to be an inducer of rodent arthritis and plasmacytomas [136]. Many natural isoprenoids like vitamin E and squalene are known also for their beneficial effects on immune system [139, 140]. However, squalene, a natural triterpene from shark liver oil which is used in MF59 adjuvant formulation, has been linked to adverse effects in rodents. From this prospective, we considered revisiting the utility of terpenoids as more versatile vaccine adjuvants by introducing chemical modifications that improve their safety and increase their adjuvanticity. During our ongoing study, we developed a series of phytol-based adjuvants by chemical modifications of phytol, the hydrophobic tail of chlorophyll. Our reports show that phytol and

its derivate phytanol (PHIS-01) are effective adjuvants in inducing robust and broadly effective immune responses against both external threats such as environmental bacterial hazards, as well as internal threats due to cancer or autoimmunogens [141, 142]. However, compared to phytol, PHIS-01 exhibits superior adjuvanticity but with less toxicity [141, 142]. Thus, PHIS-01, which is generated by removal of the only double bond, present in phytol, is a more effective adjuvant. This observation led us to question the importance of the polar hydroxyl group in PHIS-01. In this study, we modified the –OH group by amination producing phytanyl amine (PHIS-02) and by mannosylation producing phytanyl mannose (PHIS-03). The objectives are two-fold: (1) to assess their safety and efficacy as vaccine adjuvants, (2) to examine how changes at the polar terminus of PHIS-01 affect adjuvanticity. These and other ongoing modifications have been conceived in order to improve and develop effective terpenoid adjuvants with broad specificity with little toxicity.

Specific Aims

As discussed above, we will examine the adjuvant activity of two phytol derivatives named PHIS-02 and PHIS-03. The first objective is to assess their safeties and efficacies as vaccine adjuvants. The second objective is to examine the basic physicochemical proprieties necessary for a safe and efficient oil-in-water adjuvant by the assessing the structure-function relation in adjuventicities of the different phyol derivatives (PHIS-01, PHIS-02, PHIS-03).

First aim: The first aim of this study is to assess the safety and efficacy of these newly developed Phytol-based adjuvants. We will compare PHIS-02 and PHIS-03 with PHIS-01 and other commonly used adjuvants and determine to what extent their adjuvanticities depend on apoptotic/necrotic processes for activation of antigen-presenting cells, and subsequently the

acquired immune response. Using two soluble, potentially autoimmunogenic proteins, ovalbumin and a hapten-protein conjugate phthalate-keyhole limpet hemocyanin (KLH), we will assess titer, specificity, and isotypic profiles of antibody response as well as T cell proliferation and cytokine production.

Second aim: The second aim of the study is to compare the immune-competence of phytol-based adjuvants with alum's in terms of cytokine/chemokine microenvironments that are generated. Protein expressions and RT-PCR inflammasome arrays will be used to examine the cytokine/chemokine of mice peritoneal exudates (PE) at different time points after injection. Four issues will be addressed: (1) whether phytol-based immunostimulants effect bioresponse modifiers in the same way as alum does; (2) whether different protein antigens impact differently; (3) whether the antigens and adjuvants together as in vaccine formulations evoke the same or selectively magnify the effects on cytokine milieu; and (4) whether phytol compounds involve inflammasome pathways as their primary modes of action.

Third aim: During the third aim of this study, we will examine whether Phytol based adjuvants can be employed as a generic approach to contain or reverse the aggravating effects of preexisting autoimmune responses such as lupus-like autoimmune response induced by phthalate. We will focus our effort on assessment of immune parameters associated with choice of adjuvant which may down regulate or aggravate autoimmune disease activity induced by phthalate in autoimmune prone mice NZB/w f1 mice: 1) Titer and isotype of anti-DNA response to phthalate in combination with phytol-based adjuvants compared to alum, or the clinically approved adjuvants, SIS-H and SIS-M provided by Cook bio-Teck and 2) Assess alteration of the host microenvironment in terms of the chemokines milieu, Th1/Th2 balance,

and systemic pro/inflammatory cytokine balance. Taken together, these parameters may play important role in ameliorating the overall effect of the vaccine by changing the course of immune response.

Figure 1.

Stranger and Danger Model.

Figure adapted from [38].

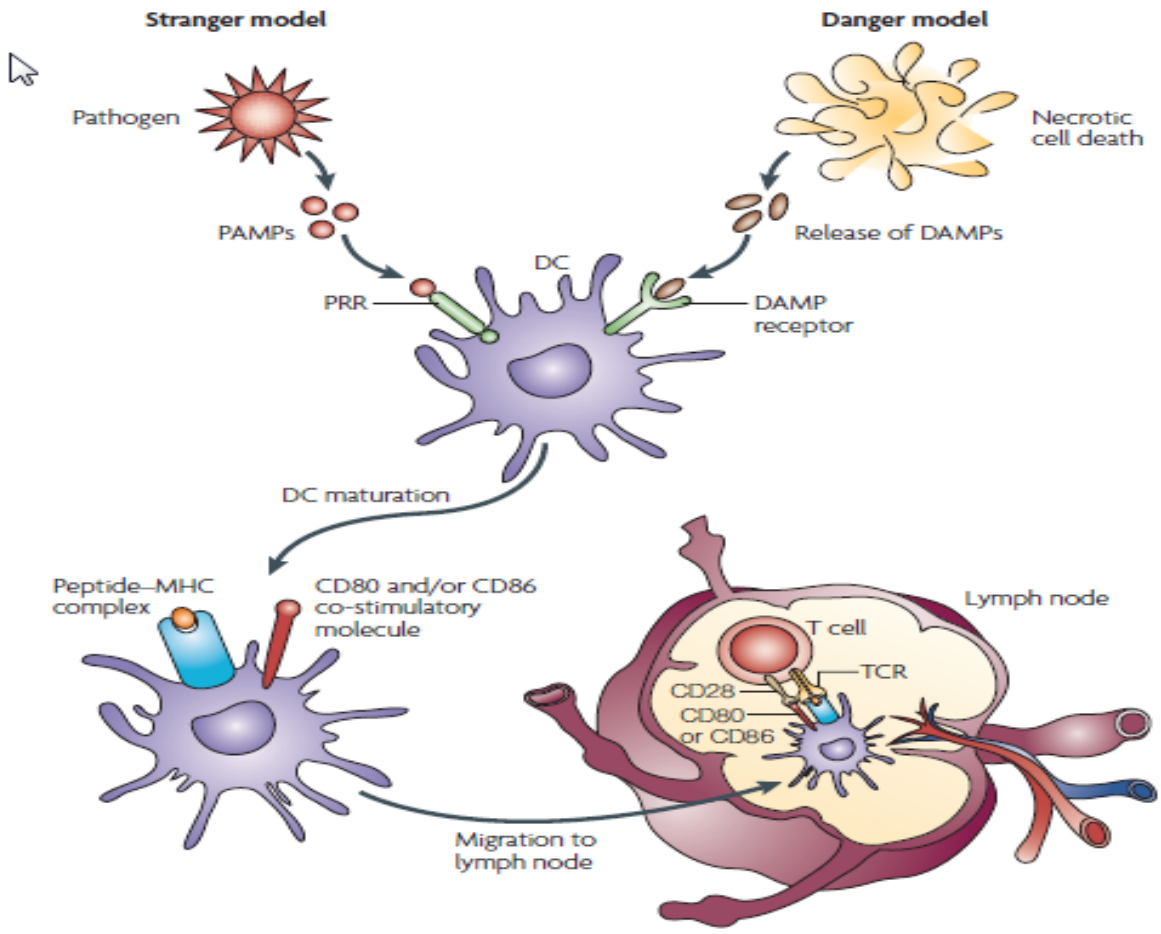

Figure 2.

Diagram of Ligands Recognized by TLR Family and Their Signaling Pathways.

Figure adapted from[143].

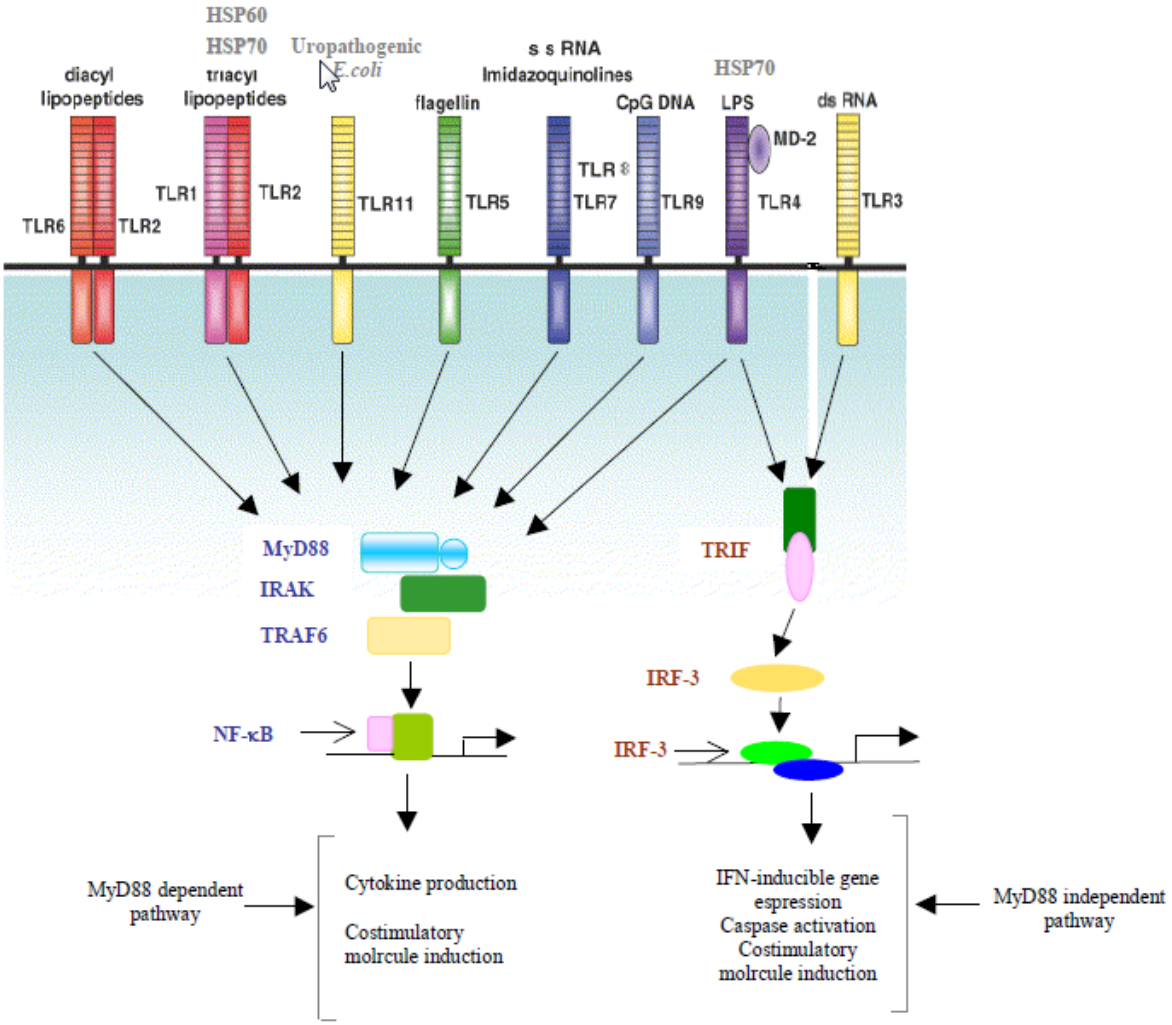

Figure 3.

Human and Mouse NLR Family Members.

Figure adapted from[53].

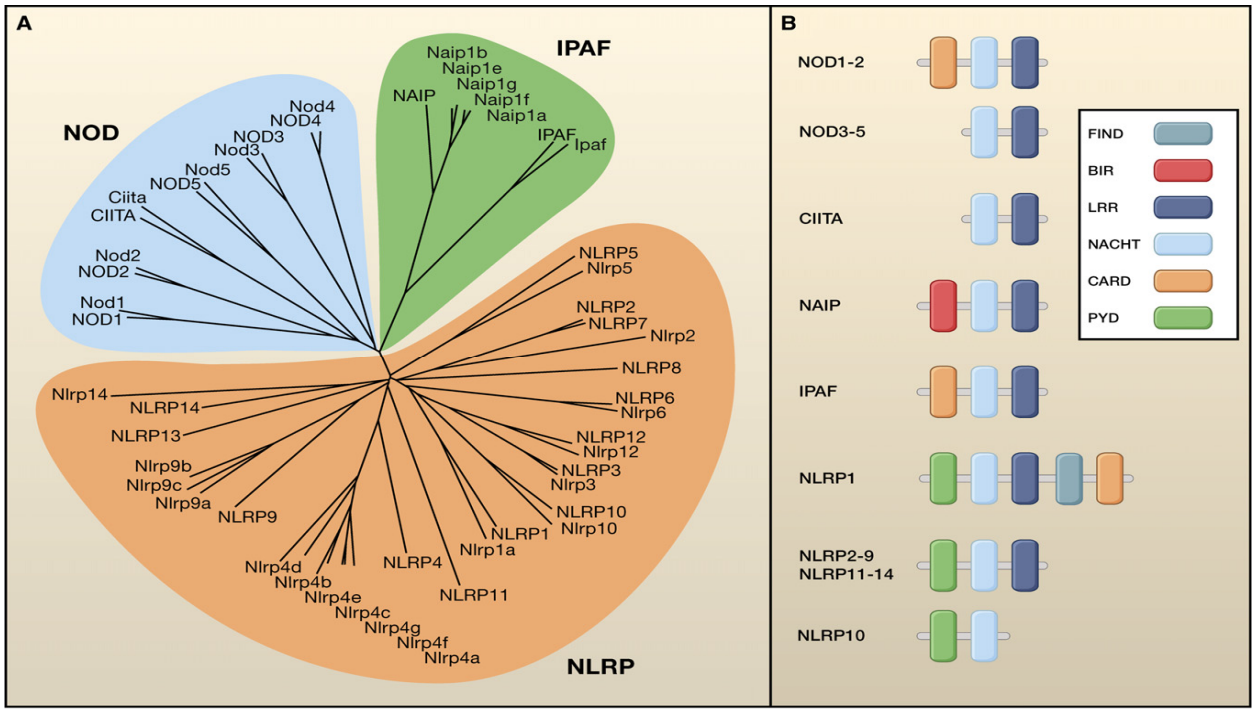

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Figure 4.

Mechanisms of Activation of NLRP3 Inflammasome.

Figure adopted from [53].

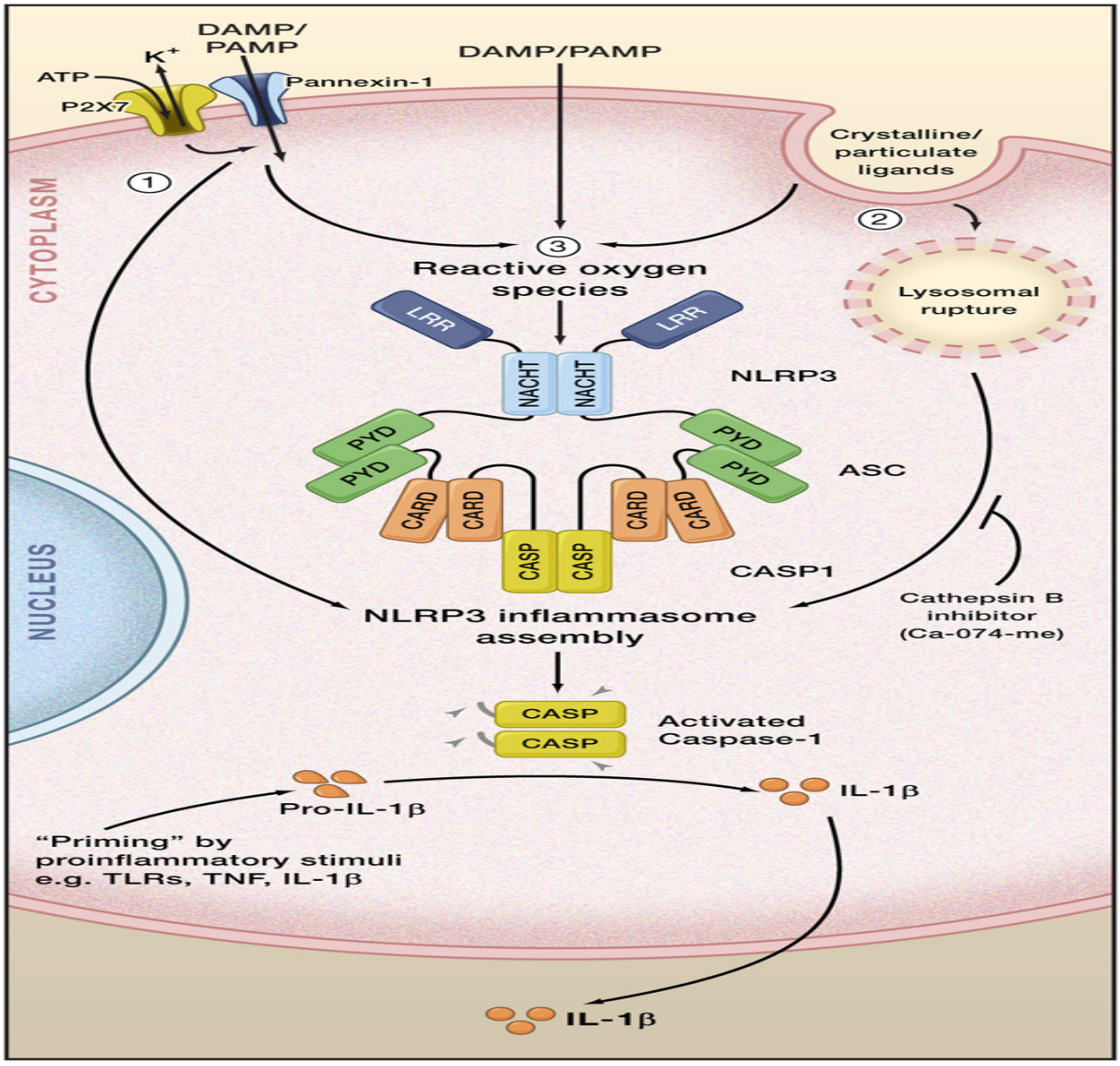


Figure 5.

Differentiation of Different T Helper Subset.

Figure adapted from [144].

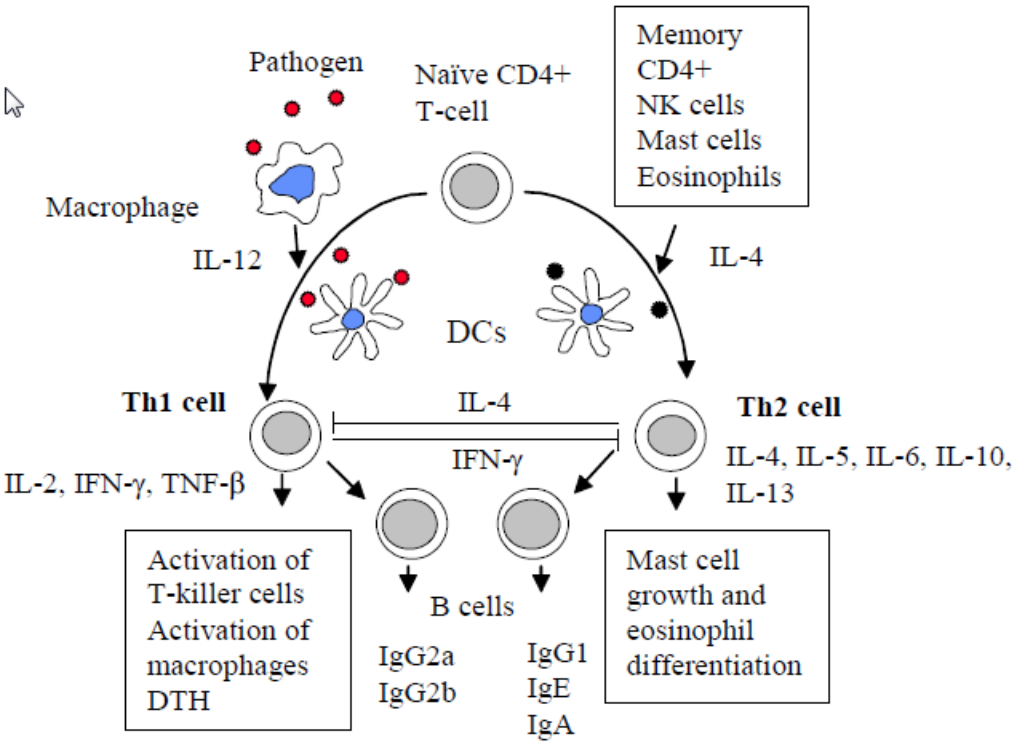


Figure 6.

List of Chemokines and Chemokine Receptors: Tissue Distributions, and Ligands.

Table adapted from[145].

Chemokine	HCC-1 MCP-2 MCP-3 MIP-1α RANTES	MCP-1 MCP-2 MCP-3 MCP-4 MCP-5	MCP-3 MCP-4 Eotaxin Eotaxin-2 RANTES	MDC TARC	RANTES MIP-1α MIP-1β MCP-2	MIP-3α (LARC)	ELC SLC	I-309, TCA-3	TECK	MCP-1 MCP-3
Chemokine receptor	CCR1	CCR2	CCR3	CCR4	CCR5	CCR6	CCR7	CCR8	CCR9	CCR10
Receptor expressing cell type	Monocyte Dendritic cell (immature) Th1 T cell Neutrophil Eosinophil Mesangial cell	Monocyte Dendritic cell (immature) Basophil T cell Natural killer cell	Eosinophil Basophil Th2 T cell Dendritic cell	Dendritic cell (mature) Basophil Th2 T cell	Th1 T cell Dendritic cell (immature) Monocytes Natural killer cell	Dendritic cell (immature) T cells B cells	Dendritic cell (mature) T cell (naive) B cell	Monocyte	T cell	

HCC, hemofiltrate CC chemokine; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated upon activation, normal T cell expressed and secreted; MDC, macrophage-derived chemokine; TARC, thymus and activation-regulated chemokine; LARC, liver and activation-regulated chemokine; ELC, EBI1 ligand chemokine; SLC, secondary lymphoid tissue chemokine; TECK, thymus-expressed chemokine.

CHAPTER 2

MATERIALS AND METHODS

Mice

Female BALB/c, C57 BL/6, and NZB/W F1 mice 6-8 weeks of age were used throughout this study. C57 BL/6 mice and NZB/W F1 mice were purchased from Jackson laboratory. All animals were housed in the animal facility of Indiana State University according to principles of laboratory animal care (NIH publication 85 23) followed under a specific protocol approved by the Institutional Animal Care and Use Committee (ACUC) of Indiana State University.

Chemicals

Reagents used in this study were from the following sources: Ortho-phthalate (Pfaltz and Bauer, Inc., Waterbury, CT); ß-cyclodextrin, calf thymus DNA, rabbit anti-mouse immunoglobulin–horseradish peroxidase (Ig-HRP) reagent, o-phenylene diamine (OPD), Annexin V apoptosis kit, methylated bovine serum albumin (mBSA), OVA and BSA (Sigma Chemical Co., St. Louis, MO); KLH (Calbiochem, CA); Dulbecco's modified Eagle's minimal essential medium (DMEM), isotyping kit (Invitrogen., Carlsbad, CA); polyvinyl 96-well flat bottom plates (Falcon). CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS assay kit, Promega, Madison, WI), cytoTox96 non radioactive cytotoxicity kit and Wizard® SV Genomic DNA Purification System kit (Promega, Madison, WI).

Adjuvants Used

Adjuvant used during this current study are: Phytol, ALUM, squalene, Complete Freunds adjuvant (CFA), and incomplete Freund adjuvant (IFA) (Sigma Chemical Co. , St. Louis, MO); PHIS-1 PHIS-02 and PHIS-03 (US patent pending) were obtained by chemical modification of phytol according to the literature [146-149]. All reagents and chemicals used for the synthesis were ACS grade, and all new compounds gave satisfactory NMR, IR and MS data. Adjuvants; SIS hydrated and SIS melatonin, were obtained from Cook Biotech, West Lafayette, IN.

Immunizations

Ortho-phthalate-protein conjugates were prepared by azo-coupling the diazotized 4-aminophthalic acid (disodium) to KLH as described by Ghosh et al [150]. Briefly, 200 µL of phthalate-KLH (100 µg/mice), or ovalbumin (100 ug/mice) was emulsified in equal volumes of either complete or incomplete Freund's adjuvants (CFA) or (IFA), PHIS-01(43 mg/mice) PHIS-02 (2.5 mg/mice), PHIS-03 (5mg/mice), Alum or squalene by vigorously mixing a few times with a syringe and vortex. The emulsion prepared was given intraperitoneally in a volume of 400µL to six to eight-week old mice (six mice per group). Mice are given two booster injections at 10 day-interval, and bled 5 days after each immunization through retro-orbital veins. The parallel control groups of mice were immunized with ortho-phthalate-KLH in PBS.

To evaluate the effect of SIS adjuvant in augmenting the immune response to phthalate-KLH conjugate or ovalbumin (OVA), we immunized mice as follows. 200 µL of phthalate-KLH (100 µg/mice), or, OVA (100 µg/mice) were emulsified in experimental adjuvant SIS-H or SIS-M provided by SIS Biotech as follow: 200 µl of antigen (100µg/mL) + 5mg (SIS-H or

SIS-M) in 250 mL PBS/15% Arlacel A. For parallel comparison commercial adjuvants such as ALUM, CFA/IFA or no adjuvant were used. Adjuvants were used as described by manufacturer (sigma).

Vaccine preparations were injected into mice either intra-peritoneally (IP) or subcutaneously (SC). Mice were given two booster immunizations at 10 day intervals and were bled under anesthesia through retro-orbital veins 5 days after each immunization.

Enzyme-Linked Immunosorbent Assays (ELISA)

Direct or sandwich ELISA were performed to assess different immune responses as follow:

Assessment of Serum Levels of Anti-Phthalate, Anti-OVA and Anti-DNA Antibodies

Determination of levels of anti-phthalate and anti-ova antibodies was assessed by enzyme linked immunosorbant assays (ELISA). ELISA plates were coated for 2 hrs at 37 ºC with 50 µL of 10 µg/mL of either phthalate conjugated to BSA or OVA. The plates were washed four times with PBS containing 0. 01% Triton X-100, blocked overnight with 1% BSA and washed again. Various dilutions (10^1-10^5) of test sera (individual mice sera) from normal and immunized mice were added in triplicate to the plates, and incubated at 37°C for 1 hr. Following incubation and after washing four times with PBS/Triton X-100, rabbit anti-mouse immunoglobulin-horse-raddish peroxidase (HRP) (50 µL) (at 1: 3000 dilutions) was added. Plates were incubated for 1hr and washed again. The rabbit anti-mouse immunoglobulin-HRP was detected by addition of o-phenyl diamine (OPD). The reaction was stopped by adding 50 µL of 10% H_2SO_4, and the intensity of color was determined at optical density (OD) 490 nm.

To determine anti-DNA antibodies, ELISA plate were pre-coated for 2 hrs at 37 ºC with 50 µl of methylated-BSA (50 µg/mL). The plates were washed four time, and coated with calf thymus DNA (10 µg/mL), and incubated for 2hrs at 37C, and ELISA experiment was carried as described above.

Antibody Isotypes

To determine isotypes of antibodies produced (IgM, IgG1, IgG2a, IgG2b, and IgG3) individual mice sera from different groups of immunized mice were diluted to 1/100, and then tested in triplicate according to the manufacturer's protocol (Invitrogen, Carlsbad, CA or Southern biotech, Alabama).

Detection of Cytokines by Sandwich ELISA

Determination of levels of cytokine in supernatant taken from T cell proliferation assay (described below) was done by sandwich ELISA, following the protocol provided by eBiosciences (IL-4, INF γ, and IL-2 ELISA Kits). ELISA plates were coated with 100 µL/well of capture antibody and incubated overnight at 4°C. After washing the plates 5 times with ≥250 µL/well wash buffer (PBS/Tween 20), wells were blocked wells with 200 µL/well of 1X Assay diluent provided in the kit and incubated at room temperature for 1 hr. Samples were added in 100 µL/well, and incubated at room temperature for 2 hr. To detect bound cytokines, biotinylated detection antibodies specific for each cytokine were added and incubated at room temperature for 1 hr. After washing the wells as described before, 100 µL/well of Avidin-HRP was added and incubated at room temperature for 30 minutes. Wells were extensively washed; substrate solution (100 µL/well) was added to each well. The plates were incubated at room temperature for 15 min. The reaction was stopped by adding 50 µL of 10% H_2SO_4 to each well, and the absorbance at 450 nm was determined. Absorbance measurements were

translated to pg/mL by extrapolation from a standard curve prepared using purified recombinant cytokines run in parallel with each assay.

T cell Proliferation Assay

Mice were immunized by i. p. administration of 100 μg ortho-KLH emulsified in phytol, PHIS-01, PHIS-02, or PHIS-03. The control group received only Ortho-Phthalate-KLH in PBS. Fourteen days later mice were sacrificed and spleens were removed. Single cell suspensions of splenocytes were seeded into 96 well tissue cultures at $2x10^5$ cells /well in 100 μl of RPMI1640/10% Calf serum (CS), and incubated for 72 hrs with antigen at10 μg/mL. Cellular proliferation was determined by measuring conversion of MTS into formazan by the reductase system of the living cells. This was done using the MTS assay kit (Promega) according to the manufacturer's protocol. Prior to the addition of MTS reagent, 100 μL of supernatants were removed from each well and stored at -70 °C for cytokine (IFN- γ, IL-4, IL-2) assay by sandwich ELISA using antibodies and protocol of e-Bioscience.

Evaluation of Apoptotic and Necrotic Adjuvants Activity

Cell Lines

Three mouse B-lymphoma/myeloma lines, 2C3, A20 and Sp2/0-Ag14 (ATCC) were cultivated in their respective media. 2C3 cells were grown in DMEM with 10% horse serum, and A20 cells and *Sp2*/0-*Ag14* cells were grown in RPMI1640 medium supplemented also with 10% horse serum.

Preparation of Phytol and Phytol Derivatives for *In-vitro* Assay

Phytol or phytol derivatives used in this study are hydrophobic compounds. Two methods were used to solubilize our test agents. In the first method, Phytol, PHIS-01, PHIS-02 and PHIS-03 were solubilized in 5% PBS/DMSO. The second method involves forming an

inclusion complex of the compounds with ß-cyclodexterin (ß-CyD)[151]. Phytol, PHIS-01, PHIS-02, or PHIS-03 (final concentration 2mM) was added to 4 mM ß-CyD solution in PBS and stirred at room temperature for 4 days. The crystalline inclusion complexes, which precipitated out of solution after 10 min centrifugation, were solubilized in a solution of 2% DMSO + 2% Ethanol in deionized H_2O and stored at 4C until further used.

MTS Assay for Lymphoma Cells

Cell viability of tumor cells was measured using the MTS assay measuring the extent of conversion of MTS into MTT farmazan by the reductase system of the living cells. Briefly 2C3, A20 or SP2ag 14 (10^5 cells) were seeded in 96 well plates in a total volume of 100 µl culture medium (DMEM, 10% FBS, penecillin/strepamycin), then a small volume of the test agent prepared either in DMSO or β-cyclodexterin as described above (phtyol, PHIS-01, PHIS-02, or PHIS-03) was added to give the final concentration specified in the text, and the mixture was incubated for 24hr at 37C. 20 µl of dye reagent provided in the KIT was added to plates and incubated for 2 hr at 37C 5% CO2, and color development was measured at 490 nm.

LDH Release Assay for Lymphoma Cells

To estimate the necrosis activity of Phytol, PHIS-01, PHIS-02 and PHIS-03, the LDH release assay was used in this study. LDH, released from lysed cells after incubation with different test agents, was measured using the cytoTox96 non radioactive cytotoxicity kit (Promega). LDH present in culture supernatants catalyzed the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of color formed is proportional to the number of lysed cells. Briefly, 2C3 (10^5 cells) were seeded in 96 well plates in a total volume of 100 µL culture medium; then a small volume of the test agent was added to provide the final concentration specified in the text. Then the mixtures were incubated for 24 hrs at 37 °C.

Measurement of the amounts of LDH release was conducted as described by the manufacturer. Farmazan the end product of the reaction was quantified by record fluorescence at 560/590nm.

DNA Fragmentation Assay

To estimate cell death, 2C3 cells were seeded in 6 well tissue culture plates at 10^6 cells per mL in a total volume of 3 mL. The test agents were added to give the final concentrations specified in the text, and the cells was incubated for 24 hrs at 37 °C. Cells were washed twice with PBS and DNA was isolated from cellular pellets using a Wizard® SV Genomic DNA Purification System kit. Equal quantities of DNA were loaded into wells of a 2% agarose gel and electrophoresed in TAE buffer for 45 min at 90 volt. The gel was stained by Ethidium bromide solution (10 mg/mL in TAE buffer) for 10 min and distained for 10 min in water. A Gel picture was taken under UV light digital camera.

Fluorescence Microscopic Analysis using Annexin V and PI Staining

To evaluate apoptotic cell death associated with phytol-based adjuvants, 2C3 cells were seeded in tissue culture plate at10^6 cells/ mL in a total volume of 1mL. The test agents were added to obtain the final concentration specified in the text, and the mixtures were incubated for 24 hr at 37 °C. Cells were centrifuged and washed twice with PBS and resuspended in 1X Ca^{2+} enriched binding buffer (Annexin V apoptosis kit, Sigma), at a concentration 10^6 cells/mL. Then, 5 µL of annexin V-FITC (1µg/mL) and 10 µL of propidium Iodide (1 µg/mL) were added to each cell suspension (500 µl, $5x10^5$ cells). Cells were stained for 10 min at room temperature, protected from light, then were mounted on glass slides and examined under fluorescence microscope.

Cytokines and Chemokines Arrays

Collection of Peritoneal Cells and Lavages

BALB/c mice (n=3) immunized with inoculums containing either KLH or OVA in combination with alum, PHIS-01, PHIS-02, PHIS-03, SIS hydrated, or SIS melatonin as described above were sacrificed at 2 hr, 24 hr and 72 hr after injection. Thereafter, 3 mL of PBS was used to collect peritoneal lavage using a 19G needle, then the collected samples were pooled and centrifuged (4°C, 400 g, 10 min). Supernatants were collected for cytokines and chemokines analysis. Peritoneal cells were washed twice with PBS and used for profiling inflammation-related genes expression.

Determination of Cytokines and Chemokines Secreted in the Peritonea

Cytokines and chemokines in peritoneal fluids were assessed using RayBioTecK mouse inflammatory Cytokine Array II (Raybioteck, Inc) following the manufacturer's instructions. Briefly, cytokine array membranes provided were blocked in 2 ml of blocking buffer for 30 min and then incubated with 1 ml of undiluted samples at 4^{o}C for overnight. Samples were then decanted off, and the membranes washed three times with wash buffers. Membranes were incubated in diluted biotin-conjugated primary antibodies (1: 250) at room temperature for 2 hr, washed and exposed to horseradish peroxidase-conjugated streptavidin (1:1000) for 1 hr. This was followed by treatment for 2 min with 500 µl of peroxidase substrate in the dark, and exposure of the membranes to X-ray film (Kodak X-OMAT AR film). Subsequently, the films were developed and signal intensities of all spots were analyzed to figure out relative expression indices of cytokines released.

Cytokines and Chemokines Quantification

Cytokine signal intensities were quantified and analyzed with Image J software published by at the NCBI website [152]. Positive controls and negative controls at six spots were used to normalize the results in different membranes. For each spot, the net optical density level was determined by subtraction of the background density from the sample density and then divided by the density of the positive control. The results were expressed as relative intensity (RI) in percentage to positive control.

Inflammasome Array

RNA Isolation

Total RNA isolation was done according to the manufacturer's (Ambion, Austin, TX). All reagents used are provided in the Kit. Peritoneal cells isolated (Approximately 10^6) as described above were washed twice in 5mL PBS and centrifuged for 5 min. Cells pellets were resuspended by vortexing vigorously in 300 μL lysis solution and 150 μL of 100% ethanol was added. Samples were mixed thoroughly by pipeting a few time and vortexed briefly. Lysate/ethanol mixtures (up to 150 μL) were loaded onto a micro-filter cartridge assembly and centrifuged for 30 sec at 13200 RPM. This procedure was repeated with additional aliquots until the entire sample has passed through the filter. Filter was washed with 180 μL wash solution 1 and centrifuged for 30 seconds at 13200rpm. Filter was washed again twice with 180 μL wash solution 2/3, and dried by centrifuging for 2 min at maximum speed. Then, micro-filter cartridges were transferred to new elution eppinderof tube, and 20 μL of elution solution, preheated to 75°C, to the center of the filter. Filter was stored for 1 min at room temperature, and then centrifuged for ~30 sec to elute the RNA. This step was repeated with a second 20 μL aliquot of preheated elution solution. After measurement of RNA concentration in each

sample, 1 µg of RNA was treated with DNase provided in RT First Strand cDNA Kit (SABiosciences, Frederick, MD) to eliminate genomic DNA contamination as follow:

Total RNA 25 ng to 5 mg

GE (5X gDNA Elimination Buffer) 2 µL

H2O to a final volume of 10 µL

Contents were mixed gently by pipeting and incubated at 42 ºC for 5 min, then chilled on ice until later use. RNA quality was assessed spectrophotometrically; all samples had 260/280 ratios above 2.0 and 230/260 ratios above 1.7.

Synthesis of cDNA

cDNA synthesis was done using RT First Strand cDNA Kit (SABiosciences, Frederick, MD). Briefly experimental cDNAs were prepared as follow:

RNA (1µg):	10 µL
BC3 (5X RT Buffer 3):	4 µL
P2 (Primer & External Control Mix):	1µ L
RE3 (RT Enzyme Mix 3):	2 µL
H2O:	3 µL
Final Volume:	20 µL

Samples were mixed well by gentle pipeting, and cDNA synthesis was performed using a Bio-Rad ALD1233 Peltier Thermal Cycler (Hercules, CA). Samples were incubated at 42°C for exactly 15 min, and immediately the reaction was stopped by heating at 95°C for 5 minutes. Then, 91 µL of H_2O was added to each 20 µL of cDNA synthesis reaction and mixed well. The finished First Strand cDNA synthesis reaction was kept on ice until the next step or stored at -20°C.

Real-Time qPCR

Real-Time Polymerase chain reaction (PCR) was performed using RT2 Profiler PCR inflammasome array PAMM-097 (SABiosciences, Frederick, MD) and RT2 Real time qPCR SYBR Green/ROX MasterMix2 (SABiosciences, Frederick, MD). Five house-keeping genes, RT controls, and PCR controls were included in the PCR array. Briefly experimental cocktails were prepared as follow:

2X SABiosciences RT2 qPCR Master Mix:	1350 µL
Diluted First Strand cDNA Synthesis Reaction**:**	102 µL
H2O:	1248 µL
Total Volume:	2700 µL

Then, 25 µL of the Experimental Cocktail was added to each well of the 96 well PCR Array. PCR Array plates were tightly sealed with optical thin-wall 8-cap strips and Centrifuged for 1min at room temperature at 1000 g to remove bubbles. Real time qPCR was performed on a Stratagene Mx3000P cycler using the following cycling program:

Cycles	Duration	Temperature
1	10 minutes	95℃
40	15 seconds	95℃
	1 minute	60℃

After the reaction stopped, threshold value was manually defined using the Log view of the amplification plots. Threshold value was placed above the background signal but within the lower one-third phases of the amplification plot. The thresholds value is kept the same across all PCR Arrays used. Cycle threshold values (Ct) for all wells were analyzed with the SABiosciences Web-Based PCR Array Data Analysis provided by SABiosciences. Gene

expressions were normalized to all five house-keeping genes included in the array and calculated as average log2 ratio. The results are expressed as fold change value compared to none adjuvant-treated group.

Statistical Analysis

Paired Student's t-test, independent student's t-test, and one-way ANOVA (SPSS software) were used to determine statistical significance. Levels of $p < 0.05$ were considered statistically significant. Data are expressed as mean ± SD.

CHAPTER 3

TOPIC 1: EVALUATION OF NEW PHYTOL DERIVATIVES IN INDUCTION AND PERSISTENCE OF SPECIFIC IMMUNE RESPONSE

Abstract

Terpenoids are ubiquitous natural compounds that have been shown to improve vaccine efficacy as adjuvants. To gain an understanding of the structural features important for adjuvanticity, we studied compounds derived from a diterpene phytol and assessed their efficacy. In a previous report, we showed that phytol and one of its derivatives, PHIS-01 (a phytol-derived immunostimulant, phytanol), are excellent adjuvants. To determine the effects of varying the polar terminus of PHIS-01, we designed amine and mannose-terminated phytol derivatives (PHIS-02 and PHIS-03, respectively). We studied their relative efficacy as emulsions with soluble proteins, ovalbumin and a hapten-protein conjugate phthalate-KLH. Immunological parameters evaluated consisted of specific antibody responses in terms of titers, specificities and isotype profiles, T cell involvement and cytokine production. Our results indicate that these new isoprenoids were safe adjuvants with the ability to significantly augment immunogen-specific IgG1 and IgG2a antibody responses. Moreover, there was no adverse phthalate cross-reactive anti-DNA response. Interestingly, PHIS-01 and PHIS-03 influenced differentially T-helper polarization. We also observed that these compounds modulated the immune response through apoptotic/necrotic effects on target tumor cells using murine

lymphomas. Finally, unlike squalene and several other terpenoids reported to date, these phytol derivatives did not appear arthritogenic in murine models.

Introduction

Vaccine efficacy depends largely on two variables; the specific antigen(s) used and the choice of adjuvants. It is the inclusion of the latter in a vaccine formulation that significantly improves the quality and magnitude of specific immune response. Adjuvants are a diverse group of chemical compounds and vary widely in their ability to influence immune response. Selection of adjuvants is generally empirical, and the list of experimental adjuvants is growing. However, a major concern with experimental adjuvants is their potential for acute or chronic toxicity. Only a few adjuvants, including hydrophilic aluminum salts (Alum) [153] and a hydrophobic squalene-based emulsion (MF59) are licensed for human use [10, 154]. For veterinary purposes, oil-in-water emulsions such as Freund's adjuvants (FA, paraffin oil with or without mycobacterial components) and TiterMax/Ribi's adjuvants (containing squalene) have been used [12, 155].

Several studies with oil-in-water emulsion adjuvants have shown that they help retain immunogens longer, an important parameter for good adjuvanticity. In addition, they promote activation and maturation of antigen presenting cells [156-158]. It has also been suggested that they may induce danger signals to alert the immune systems against a potential threat, but without much adverse inflammatory response [159]. Since no single adjuvant has been shown effective in every situation, there is an ever-growing need for new adjuvants. Ideally, an adjuvant should have little reactogenicity, but be broadly effective in modulating the host-immune microenvironment. However, a central issue with adjuvants is empiricism in their

selection, as mentioned earlier. This difficulty is largely due to a lack of any systematic, correlative structure-function studies.

The physicochemical properties necessary for immunomodulation by oil-in-water adjuvants are not fully understood. These emulsions function in many capacities from membrane anchoring to cell signaling [160, 161]. Many natural isoprenoids including vitamin E and squalene are known for their beneficial effects on the immune system. However, squalene (a natural triterpene adjuvant from shark liver oil) has been shown to have adverse effects in rodents [135, 162]. Furthermore, adverse effects have been reported with the naturally occurring diterpene pristane. Though an effective adjuvant, pristane has proven to be an inducer of rodent arthritis and plasmacytomas [135, 163, 164]. Similarly, phytol a natural diterpene alcohol in chlorophyll, although an effective adjuvant, produced adverse effects including splenomegaly, hepatotoxcicity, and tumor promotion in rodents [142, 165, 166].

To ascertain if these problems could be overcome, we have developed a series of phytol-based immunostimulants including PHIS-01, PHIS-02 and PHIS-03 by chemical modifications of phytol (US patent pending 11/295131). In previous reports, we established that PHIS-01 (phytanol) is an effective adjuvant [141, 142, 167]. It is stable and has no detectable toxicity. It can enhance both humoral and cell-mediated immunity, and can exert ameliorating effects in lupus-prone NZB/WF1 mice. The efficacy of PHIS-01 led us to explore the importance of its polar alcoholic group, PHIS-02 (phytanylamine) was produced from PHIS-01 by conversion to the bromide and Gabriel synthesis to give the amine [146, 147]. PHIS-03 was prepared by mannosylation of PHIS-01 with pentaacetylmannose using the trichloroacetimidate method [21, 22]. In this study, we compared PHIS-02 and PHIS-03 with PHIS-01 and other commonly used adjuvants and determined to what extent their adjuvanticity

depends on apoptotic/necrotic processes for activation of antigen-presenting cells, and subsequently the acquired immune response. Using two soluble, potentially autoimmunogenic proteins, ovalbumin and a hapten-protein conjugate phthalate-keyhole limpet hemocyanin (KLH), we assessed titer, specificity, and isotypic profiles of antibody response as well as T cell proliferation and cytokine production. We report that modified phytol-derived adjuvants significantly augment antibody response of isotypes IgG1 and IgG2a, promote effective T cell proliferation and exhibit no adverse autoimmune anti-DNA response. We also noted that these phytol derivatives function by activation of antigen-presenting cells involving apoptotic/necrotic effects on target cells. In the accompanying paper, we determined how apoptotic/necrotic effects influence expression profile of inflammation-related cytokine and chemokine genes.

Materials and Methods

Animals

Female BALB/c and C57Bl/6 mice, 6-8 weeks of age were used throughout this study. All animals were housed in the animal facility of Indiana State University according to principles of laboratory animal care (NIH publication 85 23) followed under a specific protocol approved by the Institutional Animal Care and Use Committee (ACUC) of Indiana State University.

Chemicals

Reagents used in this study were from the following sources: Ortho-phthalate (Pfaltz and Bauer, Inc., Waterbury, CT); ß-cyclodextrin, calf thymus DNA, rabbit anti-mouse immunoglobulin–horseradish peroxidase (Ig-HRP) reagent, o-phenylene diamine (OPD), complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), Alum, squalene,

Annexin V apoptosis kit, methylated bovine serum albumin (mBSA) and BSA (Sigma Chemical Co., St. Louis, MO); KLH (Calbiochem, CA); Dulbecco's modified Eagle's minimal essential medium (DMEM), isotyping kit (Invitrogen., Carlsbad, CA); polyvinyl 96-well flat bottom plates (Falcon). CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS assay kit, Promega, Madison, WI), cytoTox96 non radioactive cytotoxicity kit and Wizard® SV Genomic DNA Purification System kit (Promega, Madison, WI). The phytol derivatives, PHIS-02 (phytanyl amine) and PHIS-03 (phytanol mannose) (US patent pending 11/295131) were obtained by chemical modification of phytanol as described [146-149].All reagents and chemicals used for the synthesis were ACS grade, and all new compounds gave satisfactory NMR, IR and MS data.

Preparation of Vaccine Formulation

Ortho-phthalate-protein conjugates were prepared by azo-coupling the diazotized 4-aminophthalic acid (disodium) to KLH as described by Ghosh et al [150]. Briefly, 200 µL of phthalate-KLH (100 µg/mice) was emulsified in equal volumes of either complete or incomplete Freund's adjuvants (CFA) or (IFA), PHIS-02, PHIS-03, squalene, or adsorbed on alum by vigorously mixing a few times with a syringe and vortex. The emulsion prepared was given intraperitoneally in a volume of 400 µL to six to eight-week old mice (six mice per group). Mice are given two booster injections at 10 day-interval, and bled 5 days after each immunization through retro-orbital veins. The parallel control groups of mice were immunized with ortho-phthalate-KLH in PBS.

Assessment of Serum Levels of Anti-Phthalate and Anti-DNA Antibodies

Determination of levels of anti-phthalate and anti-DNA antibodies was assessed by enzyme linked immunosorbent assays (ELISA). ELISA plates were coated for 2 hrs at 37 ºC

with 50 µL of 10 µg/mL of either phthalate conjugated to BSA or calf thymus DNA at 4°C. The plates were washed four times with PBS containing 0.01% Triton X-100, blocked overnight with 1% BSA and washed again. Serial dilutions (10-10,000-fold) of test sera from normal and immunized mice were added in triplicate to the plates, and incubated at 37°C for 1 hr. Following incubation and after washing four times with PBS/Triton X-100, rabbit anti-mouse immunoglobulin-horse-radish peroxidase (HRP) (50 µL) (at 1: 3000 dilutions) was added. Plates were incubated for 1 hr and washed again. The rabbit anti-mouse immunoglobulin-HRP was detected by addition of o-phenyl diamine (OPD). The reaction was stopped by adding 50 µL of 10% H_2SO_4, and the intensity of color was determined at OD 490 nm.

Antibody Isotypes

To determine isotypes of anti-phthalate specific antibodies produced, sera from different groups of immunized mice (2^{nd} immunization), were diluted 1:100, and tested in triplicate on phthalate-coated plates, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Detection of Cytokines by Sandwich ELISA

Determination of cytokine levels in supernatant taken from T-cell proliferation was done by sandwich ELISA, following the protocol provided by eBioscience (IL-4, INF γ, and IL-2 ELISA Kits). ELISA plates were coated with 100 µL/well of capture antibody and incubated overnight at 4°C. After washing the plates 5 times with ≥250 µL/well wash buffer (PBS/Tween 20), wells were blocked with 200 µL/well of 1X Assay diluent provided in the kit and incubated at room temperature for 1 hr. Samples were added in 100 µL/well, and incubated at room temperature for 2 hr. To detect bound cytokines, biotinylated detection antibodies specific for each cytokine were added and incubated at room temperature for 1 hr. After

washing the wells as described before, 100 µL/well of avidin-HRP was added and incubated at room temperature for 30 minutes. Wells were extensively washed; substrate solution (100 µL/well) was added to each well. The plates were incubated at room temperature for 15 min. The reaction was stopped by adding 50 µL of 10% H_2SO_4 to each well, and the absorbance at 450 nm was determined. Absorbance measurements were translated to pg/mL by extrapolation from a standard curve prepared using purified recombinant cytokines run in parallel with each assay.

MTS assays for T Cell Proliferation and Lymphoma Viability

Mice were immunized *i.p.* administration of 100 µg ortho-KLH emulsified in phytol, PHIS-01, PHIS-02, or PHIS-03. The control group received only Ortho-Phthalate-KLH in PBS. Fourteen days later mice were sacrificed and spleens were removed. Single cell suspensions of splenocytes were plated at $2x10^5$ cells /well into 96 well tissue culture plates, and incubated for 72 hrs with antigen added at a concentration of 10 µg/mL. Cellular proliferation was determined by measuring conversion of MTS into formazan by the reductase system of the living cells using an MTS assay kit (Promega) according to the manufacturer's protocol. Prior to the addition of MTS reagent, 100 µL of supernatants were removed from each well and stored at -70 °C for cytokine (IFN- γ, IL-4, IL-2) assay by sandwich ELISA as described above.

Cell viability of tumor cells was measured using the MTS assay essentially as described above.

Cell Lines

Three mouse B-cell lymphoma/myeloma lines (2C3, A20 and Sp2/0-Ag14) were used in this study. The 2C3 cells were grown in DMEM, while A20 cells and Sp2*/0*-Ag14 cells were grown in RPMI1640 medium supplemented also with 10% horse serum.

Preparation of Phytol and Phytol derivatives for Proliferations assay

Phytol and the phytol derivatives used in this study are hydrophobic compounds. They were solubilized by forming an inclusion complex of each compound with ß-cyclodexterin (ß-CyD)[151]. Briefly, phytol, PHIS-01, PHIS-02, or PHIS-03 (final concentration 2mM) were added to 4 mM ß-CyD solution in PBS and stirred at room temperature for 4 days. The crystalline inclusion complexes, which precipitated out of solution, were solubilized in a solution of 2% DMSO + 2% Ethanol in deionized H_2O.

LDH release assay

To determine whether necrotic events play any role in the adjuvanticity of the phytol derivatives, activities of cytoplasmic lactic dhydrogenase (LDH) were examined. Cells were incubated with phytol, PHIS-01, PHIS-02, or PHIS-03 and LDH was measured after lysis of the cells. This was performed using the cytoTox96 non-radioactive assay kit (Promega). Briefly, 2C3 (10^5 cells) were seeded in 96 well plate in a total volume of 100 µL culture medium, followed by addition of small volumes of the test agents as specified in the test results. Then the mixtures were incubated for 24 hrs at 37 °C. Measurement of LDH activity was done according to the manufacturer's protocol.

DNA Fragmentation Assay

To determine apoptosis-related cell death, 3 mL of 2C3 cells at 10^6/ml were incubated for 24 hr at 37 °C with the test compounds specified, in the relevant section of the result. The cells were then harvested by centrifugation and DNA isolated using a Wizard® SV Genomic DNA Purification System kit. Isolated DNA was analyzed for fragmentation by electrophoresis on 2% agarose gel in TAE buffer for 45 min.

Fluorescence Microscopic Analysis Using Annexin V and PI Staining

As further examination of cell death from longer term (24 hr) exposure to phytol-based adjuvants, FITC-conjugated Annexin V apoptosis kit (Sigma) was used to evaluate apoptosis of 2C3 cells. The experiment was similar to the one described in DNA fragmentation, above. Following incubation, an aliquot of 5 x10^5 cells were then stained for 10 min at room temperature with FITC–conjugated Annexin V and PI (1ug/mL), mounted on glass slides and examined by fluorescence microscopy. Cells showing high florescence were considered positive for apoptotic/ necrotic cell death

Statistical Analysis

One-way ANOVA and Paired Student's t-test (SPSS software) were used to determine statistical significance. Levels of $p < 0.05$ were considered statistically significant. Data are expressed as mean ± SD.

Results

Evaluation of In-vivo Toxicity of Phytol Derivatives PHIS-02 and PHIS-03

We previously reported that phytol and its hydrogenated derivatives PHIS-01 were highly effective adjuvants without any untoward effects on the host at doses required for

effectiveness [141, 142]. To assess safety and efficacy of PHIS-02 and PHIS-03, we administered these compounds as emulsions with immunogens for intraperitoneal injection in BALB/c mice. A control group received the immunogen in PBS without adjuvant. Mice were weighed prior to administration and at regular intervals for a period of one week, then sacrificed and organs such as spleens were examined for morphological and cellular changes. Table 1 shows that LD_{50} values of PHIS-02 and PHIS-03 were similar, both being between 5 and 10 mg/ mouse. No noticeable physical or behavioral changes in mice were observed, and there were no fluctuations in their body weights (Table 1). Moreover, in contrast to CFA-treated mice, the PHIS-02 or PHIS-03-groups experienced no splenomegaly at the doses used (Table 2).

Assessment of Adjuvanticity in Enhancement of Specific Humoral Response

To determine if the new phytol derivatives could function as adjuvants we administered them as emulsions with a commonly used protein ovalbumin in C57BL/6 mice. For comparison we studied alum as a reference adjuvant with or without ovalbumin. Results in Figure 7A and 7B show that all three phytol derivatives (particularly PHIS-01, PHIS-02) significantly enhanced anti-ova antibodies. The response was about 2-fold over alum, and was further amplified after a 2^{nd} booster immunization.

Next we determined the relative adjuvanticity of different phytol-derivatives in BALB/c mice for induction of phthalate-specific humoral responses, antibody isotypes, and cross-reactive anti dsDNA response. Phthalates are unique haptens present in plastics and are considered environmental hazards [168, 169]. In previous reports, we demonstrated that phthalates could induce cross-reactive anti-DNA antibodies in murine models [170, 171]. For comparison, we studied in parallel the effects of CFA/IFA, alum, and squalene. The control

group received phthalate-KLH conjugate but no adjuvant. Circulating anti-phthalate antibody response was significantly elevated in all groups except the control group. Subsequent immunizations boosted antibody response in all adjuvant-treated groups significantly over the no-adjuvant control group ($P<0.05$, Figure 8). To determine whether there was any cross-reactive anti-ds DNA response, we performed ELISA on plates pre-coated with calf thymus DNA. The results show (Figure 9) that mice immunized with ortho-phthalate plus CFA/IFA, ALUM, or squalene registered significant levels of anti-DNA antibody response compared to only the immunogen-treated group. In contrast, the new diterpene adjuvants, namely PHIS-02 and PHIS-03 did not induce detectable levels of cross-reactive anti-DNA antibodies. We previously also reported that phytol and PHIS-01 adjuvants, unlike IFA or squalene, are effective in preventing autoimmune potentials due to phthalate in different strains of mice including NZB/WF1 mice [141].

Effects of Adjuvants on Antibody Isotype Switching

The robustness of antibody response in sera of adjuvant-treated groups was further assessed in terms of isotype switching as a way to ascertain the involvement of T helper cells. The results in Figure 4 demonstrate the efficacy of both PHIS-02 and PHIS-03 was comparable to IFA, the most potent commercial adjuvant. Similar results were previously described for phytol and PHIS-01 [141]. It is apparent from the results in Figure 10 that PHIS-02 and PHIS-03 groups induced all IgG sub-classes in much the same way as IFA. However, the control group with no adjuvant treatment yielded only IgG1, while in all adjuvant-treated groups, the IgG1 level was not enhanced as much as IgG2a, IgG2b, and IgG3. Elicitation of these latter subclasses would indicate that the test adjuvants were effective in promoting T helper polarization.

Cytokines for Assessment of T-helper Polarization

As a follow-up of the above study, Th1/Th2 polarization was further evaluated in terms of their signature cytokines in the presence or absence of adjuvants. BALB/c mice were immunized once with phthalate-KLH, alone or as emulsions with different adjuvants. Re-exposure to the immunogen was performed two weeks later by incubation of splenocytes *in vitro* with the immunogen phthalate-KLH. Three days later, culture supernatants were assayed for cytokines by ELISA, and the splenocytes were used to determine proliferative responses using MTS assay kits. PHIS-01 or phytol-treated groups augmented splenocyte proliferation better than PHIS-02 or PHIS-03 (data not shown). Interestingly, as shown in Figure 11 PHIS-01 and PHIS-02-treated mice yielded significantly much more IFN-γ than those treated with PHIS-03. In contrast, the latter evoked a significantly higher level of IL-4. These results imply that hydrogenated or hydrogenated plus aminated phytol derivatives favored T helper type 1 response, while mannosylation induced T helper type 2 response.

Physiological Basis of Adjuvanticity: Evaluation of PHIS-02 and PHIS-03 in Terms of Apoptotic and Necrotic effects

Many adjuvants have been shown to induce apoptotic and necrotic cell death [172, 173]. Tissues or constituent cells undergoing apoptosis/necrosis are known to mobilize scavenger phagocytes and antigen-presenting cells (APCs) of innate immunity. The latter present MHC-bound epitopes acquired from dying cells to both helper and cytotoxic effectors T cells through cross-presentation [174, 175].

In this study, phytol and its derivatives were evaluated in vitro on murine lymphoma lines A20, 2C3 and Sp2/0-Ag14. To determine whether these adjuvants function through promotion of apoptosis/necrosis (as have been reported by mineral oil-based adjuvants) we

performed cytotoxicity assays using an MTS assay. Because of their highly hydrophobic nature and consequent near insolubility, a uniform and reproducible delivery of phytol and its derivatives to growing cells in culture proved to be difficult. To overcome this problem, we initially used a 5% DMSO solution to solubilize phytol and its derivatives and then study their effects on a BALB/c lymphoma line 2C3. Unfortunately, this did not improve solubility and reproducibility (data not shown). We then made inclusion complexes of phytol derivatives in ß-Cyclodextrin (ß-CyD) according to a technique described by Janz and Shacter [151]. This significantly improved the solubility of phytol and its derivatives. The results in Figure 12 indicate that all phytol-based adjuvants inhibited cellular proliferation in a dose dependent manner in all lymphoma models tested. Furthermore, cellular toxicity could be evaluated at significantly low doses of phytol and its derivatives delivered in ß-CyD. Cytotoxicity levels determined by LDH release assay show that treatment with 75 μM of different test agents resulted in 80 % cell lysis (Figure 13).

DNA Fragmentation Assay

To test whether the growth inhibitory effects of phytol and its derivatives were due to induction of apoptosis or necrosis, a DNA fragmentation assay was used. As shown in Figure 14, DNA fragments characteristic of apoptotic processes were observed with all test adjuvants. Oligonucleosomal-sized fragments increased with increasing amounts of phytol-based compounds.

Detection of Apoptosis and Necrosis Induced by Phytol and Phytol Derivatives by Annexin V, and Propidium Iodide (PI) Staining

To further assess whether phytol compounds exert apoptotic/necrotic effects on lymphoma cell lines, murine 2C3 tumor cells were exposed to these compounds and examined

for a characteristic marker of apoptotic process, that is display of phosphatidylserine on the cell surface, which is detected by fluorescence microscopy using Annexin V-FITC. Propidium iodide stains the cellular DNA in necrotic cells caused by disruption of membrane integrity. The results in Figure 15 show that phytol and its derivatives affected target 2C3 cells such that they underwent processes associated with advanced phase of apoptosis/early phase of necrosis. This is similar to the effects of tomatine adjuvants [173].

Discussion

The importance of adjuvants in vaccine preparations is well-known. Vaccine efficacy largely depends on adjuvants, a diverse group of chemical compounds [159]. There is however no single mechanism or chemical feature to explain the basis of their adjuvanticity. To shed light on chemical features of organic compounds that may be important for adjuvanticity, we investigated the effects of chemical modifications of phytol, a component and breakdown product of chlorophyll. We have found that phytol is an effective adjuvant in rodents [141, 176], but not without side effects [165]. This finding led us to chemically modify phytol and develop these derivatives, PHIS-01, PHIS-02, and PHIS-03. These derivatives have three chiral centers and are racemic at all three. PHIS-01 and PHIS-02 are alkanes containing a single functional group (OH or NH_2, respectively) and without the double bond present in phytol. PHIS-03 is the mannoside of PHIS-01. We report here that PHIS-01 and PHIS-02 are similar in their adjuvanticity, although PHIS-01 is more effective. Both promote Th1 type response, whereas PHIS-03 with the mannosyl moiety favors Th2 response. Thus, it appears that the nature of the polar end group in these compounds is an important selector for adjuvanticity, as exchange of the simple alcohol or amine with a mannosyl moiety as in PHIS-03 markedly alters the type of response elicited.

Chemical modification of adjuvants to improve their effectiveness is not new, but only limited reports have appeared [177, 178]. The object of these modifications has generally been to produce effective and safer products. For example, lipopolysaccharide (LPS) is toxic, but, it has an excellent ability to mobilize innate immunity and promote maturation of dendritic cells. Its modified version MPL is however a better adjuvant with reduced toxicity [178]. In *Quillaga* saponin adjuvant, introduction of an aldehyde group promotes a Th1 type cellular response against virus or cancer [177, 179, 180], whereas the deacylated saponin, lacking the aldehyde function, favors a Th2-mediated antibody response [181].

At the cellular level, adjuvants are normally considered to function by inducing limited local inflammation marked by apoptosis/necrosis of target tissues which facilitates increased antigen uptake and processing by antigen-presenting cells [172, 174]. The magnitude of this inflammatory response often depends on the dose of adjuvant. Among the phytol derivatives, PHIS-01 is non-toxic and highly effective at a wider range of concentrations (4- 44mg/mouse). PHIS-02 functions at a much lower concentration (2.5 mg/mouse), and PHIS-03 works effectively at an intermediate doses (5 mg). All phytol derivatives tested induce not only IgG1, but also IgG2a, IgG2b, and IgG3. Similar to PHIS-01, PHIS-02 induced significantly high levels of IgG2a. IgG1 and IgG2a are relatively long-lived antibodies and have the ability to activate complement-dependent cytotoxicity (CDC), and antibody-dependent cellular cytotoxicity (ADCC), preferred for protection against tumors and parasites.

We also addressed the risk of autoimmunity from phytol derivatives due to differences in immune competence among individuals. Some adjuvants (incomplete Freunds' adjuvants, squalene, alum) have been implicated in the induction of autoantibodies in non-autoimmune rodents [135, 182]. These adverse reactions may be aggravated if the vaccine is also

autoimmunogenic. To evaluate this possibility, we examined soluble auto-immunogenic proteins, ovalbumin and a hapten-protein conjugate phthalate-KLH. The latter has been shown to provoke autoimmune antibody response (specifically anti-ds-DNA) in different strains of mice, possibly because of molecular mimicry between phthalate (a plasticizer) and oligonucleotides, particularly oligo-dT_4 and oligo-dT_{10} [171]. We noted that all three phytol derivatives PHIS-01, PHIS-02 and PHIS-03 evoke little or no detectable anti-DNA immune response. Moreover, they seem to exert ameliorating effects [142]. Similar findings appeared in a previous report stating that phytol treatment decreases autoimmune response [176].

The inclusion of adjuvants in vaccines enhances secretion of different cytokines from activated T cells. Using PHIS-01 and PHIS-02, we noted a clear bias towards Th1 response as exemplified by IgG2a and INF-γ levels; whereas the use of PHIS-03 induces IL-4, indicating a shift towards Th2 response. We also note that phytol-based adjuvants are capable of inducing high rates of proliferation of in vivo primed splenocytes, particularly T-lymphocytes, as is evident from secretion of IL-2. It is interesting to recognize that in regards to IL-2 level, PHIS-02 is different from PHIS-01. Moreover, between PHIS-01 and PHIS-02 favoring Th1 response, the latter is more effective in stimulating splenic T cells.

The likely mechanism underlying the effectiveness of phytol-based adjuvants is their ability to induce apoptosis and/or necrosis in tissues adjacent to the immunization site. The induction of apoptosis followed by necrosis is known to act as the so-called “danger” signal. Since most adjuvants trigger some measure of apoptosis/necrosis, it is likely that the danger signal is an important mechanism by which adjuvants exert their effects [19]. Necrotic cells also release endogenous adjuvants such extracellular ATP and uric acid leading to the activation of the inflammasomes and mobilizing effective immunity arms [39, 183]. Whether

phytol derivatives augment immunogenicity by inducing endogenous adjuvants such as uric or hypochlorous acid remains a subject for further investigation.

In summary, the chemically modified phytol-based compounds compare well with commonly used commercial adjuvants. They evoke T-helper bias plus high-titer, complement-fixing antibodies, which are considered important in protection against cancer and microbes. The advantage of PHIS-02 and PHIS-03 is that they could be effective at lower doses than PHIS-01. Thus, it appears that phytol and PHIS-02 promote both apoptotic/necrotic pathways, whereas PHIS-01 is biased towards necrosis, and PHIS-03 is weakly biased towards apoptosis.

Finally, these phytol derivatives evoke little anti-DNA antibody response. In contrast, adjuvants like IFA and squalene appear to be autoimmunogenic [12, 184, 185]. Even diterpenes such as pristane have deleterious effects and this led to the suggestion that oil-based adjuvants may be unsuitable for immune-therapy [186, 187]. We conclude that these modified phytol compounds and in particular PHIS-01 have superior adjuvanticity over IFA, alum and squalene in that they have beneficial effects with no adverse reactogenicity including promotion of plasmacytomagenesis as is the case with pristane [141, 142].

Table1.

Determination of Safe Doses of PHIS-02 and PHIS-03 Following Intra-peritoneal in BALB/c Mice.

Test adjuvant	Dose	% Survival A week post-immunization	% Mean body weight loss				LD_{50} (mg)
			day1	day2	day4	day6	
PBS	250 µL	100	0.65	0	0	0	ND
PHIS-02	2.5 mg	100	1.2	0.65	0	0	5mg
	5 mg	50	0.63	4.55	2.97	0	
	10 mg	0	4.8	6.15	all dead		
PHIS-03	2.5 mg	100	1.86	1.76	1.27	0	9 mg
	5 mg	100	7.52	6.53	2.53	0	
	10 mg	33	10.79	12.17	15.44	6.22	
Number of Mice	6 in each category						

Table 2.

Effects of Adjuvants on Spleen, a Major Secondary Lymphoid Organ.

Mouse Group* (n=3 in each)	Spleen Weight (mg)	Cell Numbers/Spleen ($\times 10^7$)
Mouse injected with PBS	121 ±3	5.9 ±1.05
Mouse injected with Pristane	130 ±14.47	6.52 ±1.06
Mouse injected with CFA	199 ±5.57	11.88 ±1.43
Mouse injected with PHIS-02 (5mg)	128 ±13.61	8.23 ±1.62
Mouse injected with PHIS-02 (2.5mg)	120 ±18.52	7.11 ±1.02
Mouse injected with PHIS-03 (5mg)	127 ±12.06	8.75 ±0.98
Mouse injected with PHIS-03 (2.5mg)	114.33 ±10.12	8.08 ±0.8

Figure 7A.

Effects of Adjuvants on Anti-ovalbumin Antibody Response in C57 Black/6 Mice.

Mouse serum samples were collected on day 5 after 2nd immunizations with OVA plus adjuvants as described under Materials and Methods. Antibody responses were assessed using ELISA. The results represent mean ± SD (n = 6 mice per group in two separate experiments). The significance in experimental groups was determined relative to the group given antigen only (no adjuvant group) at the level of $p \leq 0.05$.

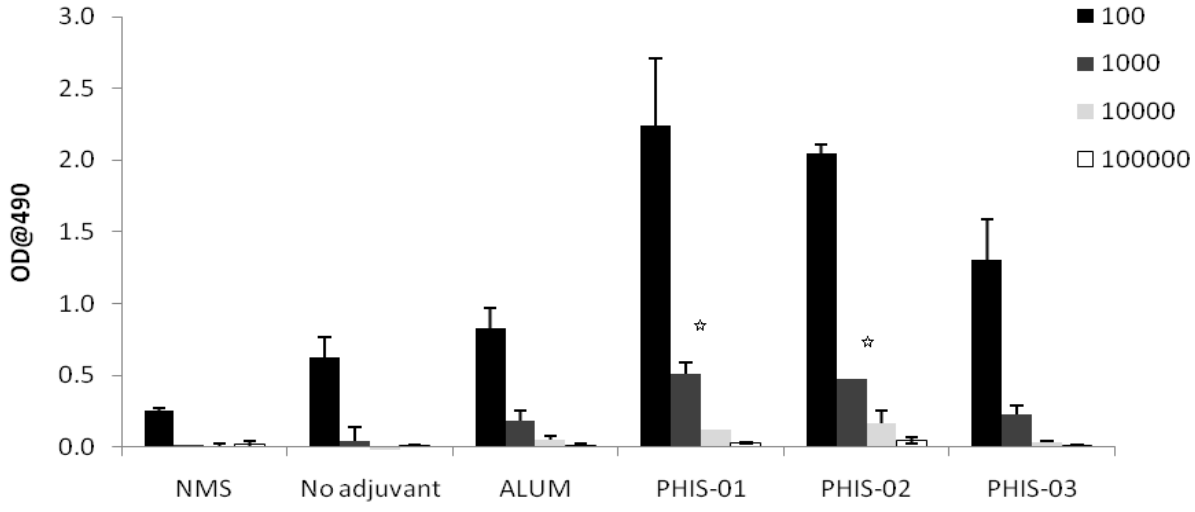


Figure 7B.

Evaluation of Anti-OVA Antibody Response Following a Repeat Vaccination

Serum samples collected on day 5 after 3rd immunizations with Ova plus adjuvants were assessed by ELISA. The results represent mean ± SD (n = 6 mice per group in two separate experiments). The significance in experimental groups was determined relative to the group given antigen only (no adjuvant group) at the level of $p \leq 0.05$.

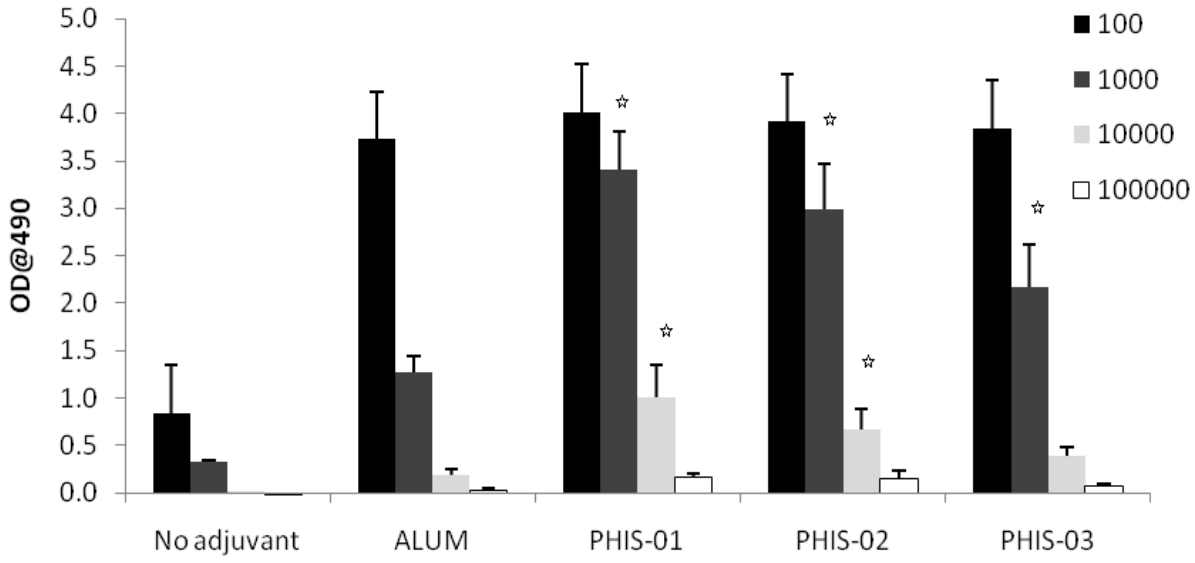


Figure 8.

Evaluation of Anti-phthalate Antibody Response in BALB/c Mice.

Mice were immunized with phthalate-KLH conjugate emulsified in different adjuvants. Serum samples were collected on day 5 after the 2nd immunizations and anti-phthalate antibody levels determined using ELISA. The results represent mean ± SD (n = 6 mice per group in two separate experiments). The significance in experimental groups was determined relative to the group given antigen only (no adjuvant group) at the level of $p \leq 0.05$.

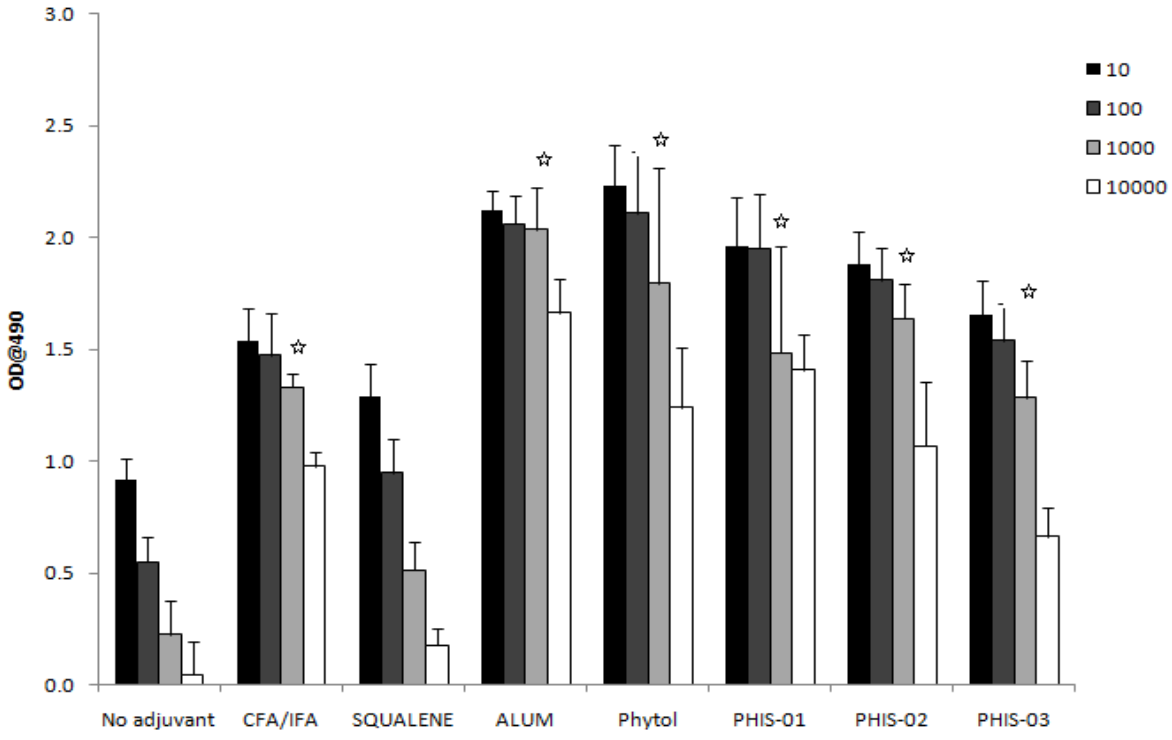


Figure 9.

Assessment of Anti-DNA Antibody Response.

BALB/c mice were immunized twice at 10 day-intervals with phthalate-KLH emulsified in various adjuvants. Their serum levels of anti-DNA antibodies were determined using calf thymus DNA-coated ELISA. The results represent mean ± SD (n = 6 mice per group in two separate experiments). The significance in experimental groups was determined relative to the group given antigen only (no adjuvant group) at the level of $p \leq 0.05$.

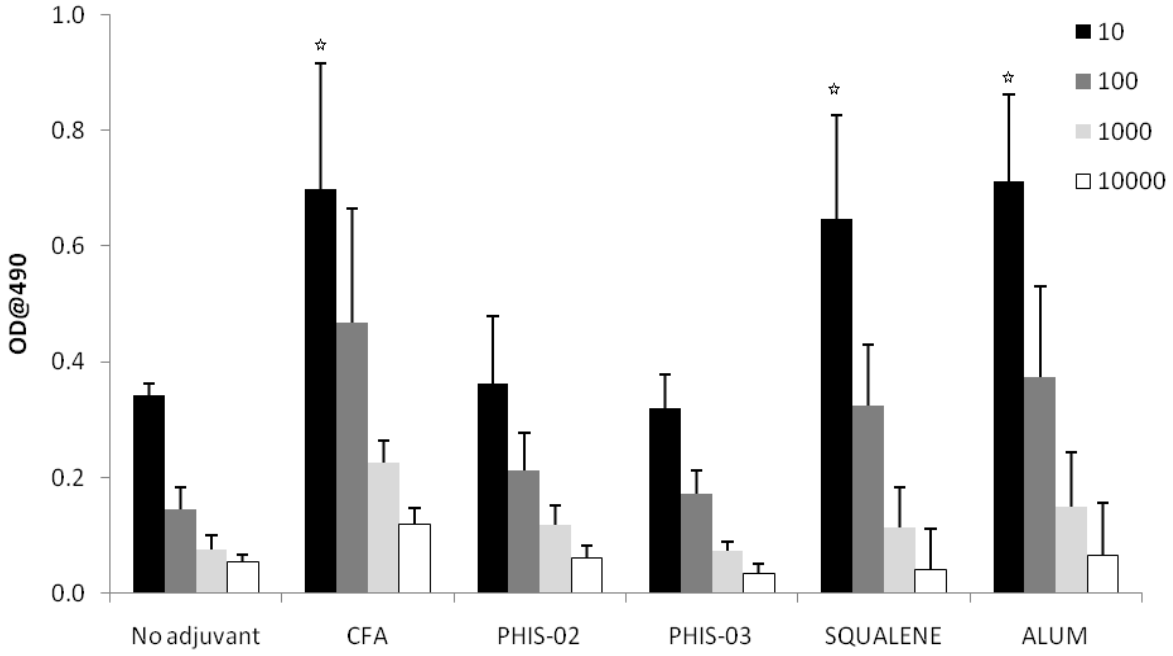


Figure 10.

Determination of IgG Sub-classes of Anti-phthalate Induced with Phthalate-KLH Conjugates in Different Adjuvants.

This was done in serum samples using commercial ELISA isotyping kits. Results represent mean ± SD (n = 6 mice per group in two separate experiments). The significance in experimental groups was determined relative to the group given antigen only (no adjuvant group) at the level of $p \leq 0.05$.

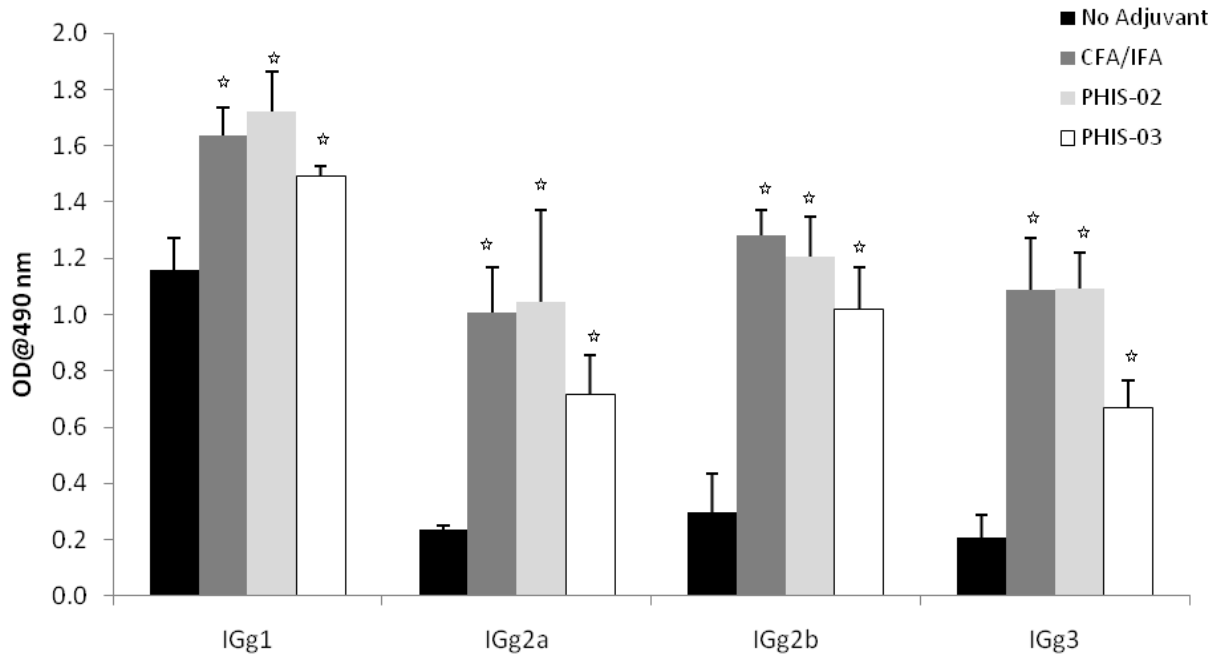
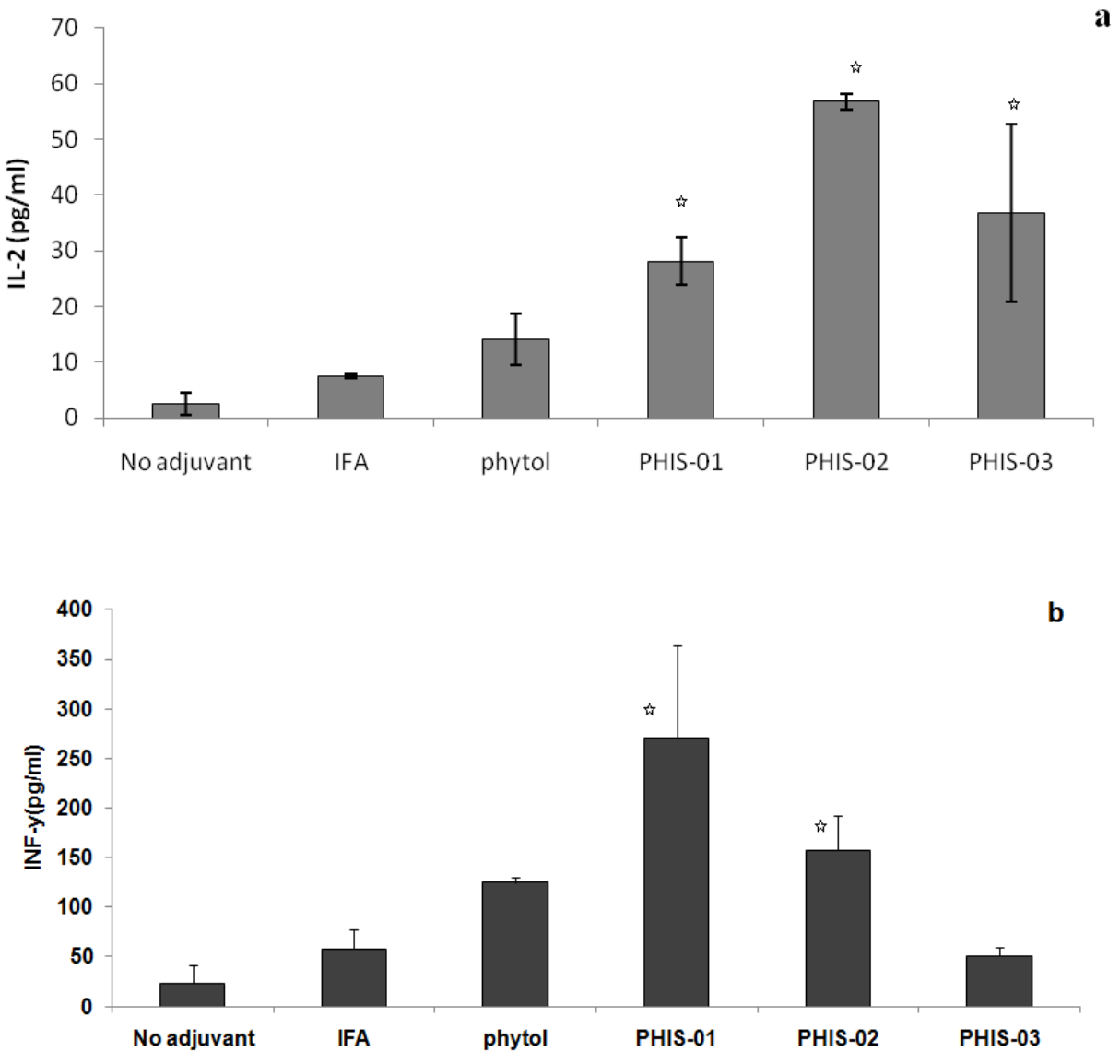


Figure 11.

Mice were Immunized i.p with Phthalate-KLH Conjugate Emulsified in Various Adjuvants. Fourteen days later spleens were removed and splenocytes were incubated in vitro with 100 μg /mL of phthalate-KLH only. Cytokines secreted after 3 day-stimulation with phthalate-KLH in vitro are depicted as (a) IL-2 produced (b) INF-γ produced; and (c) IL-4 produced.



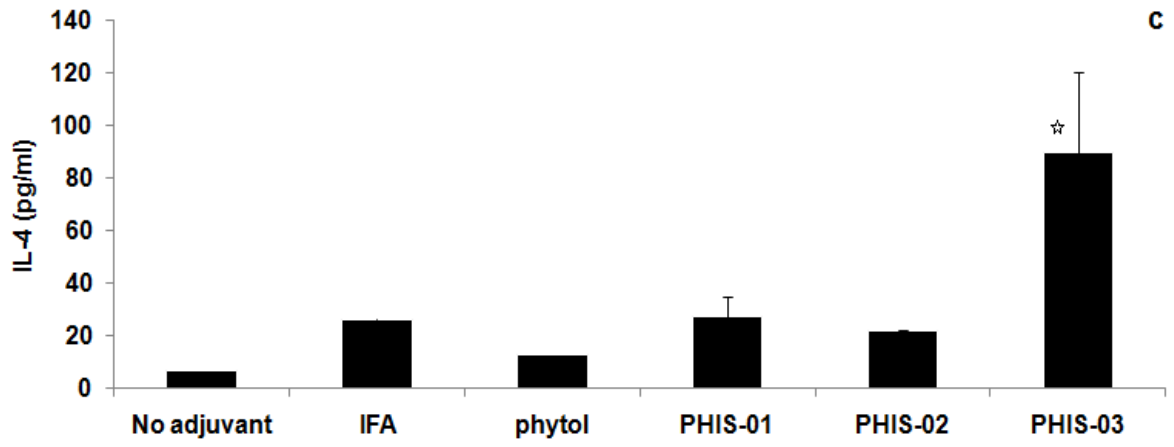
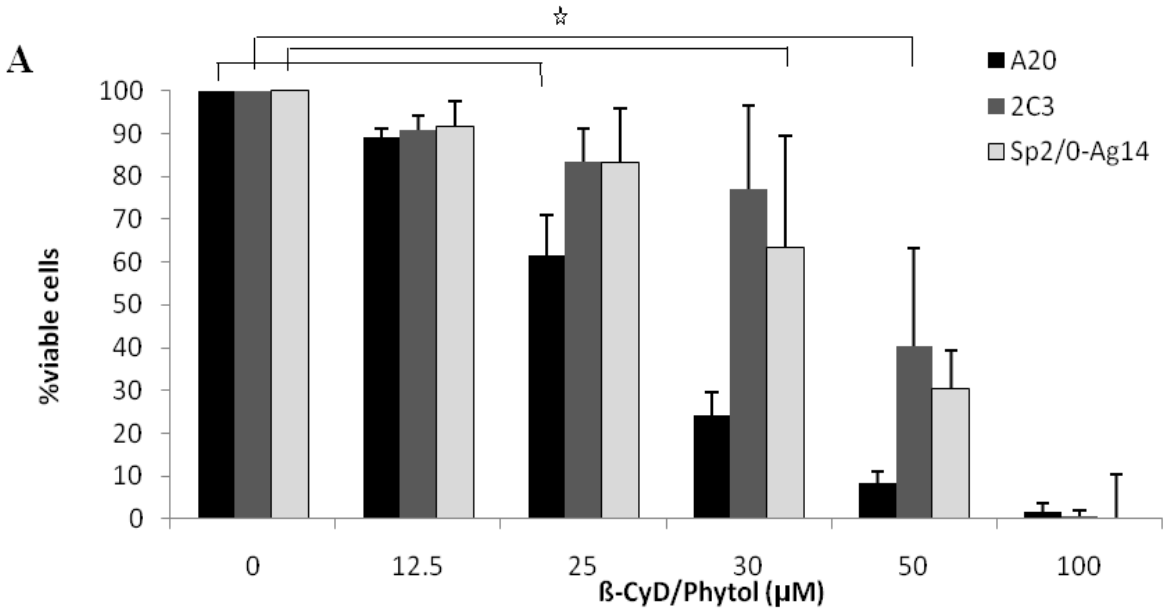
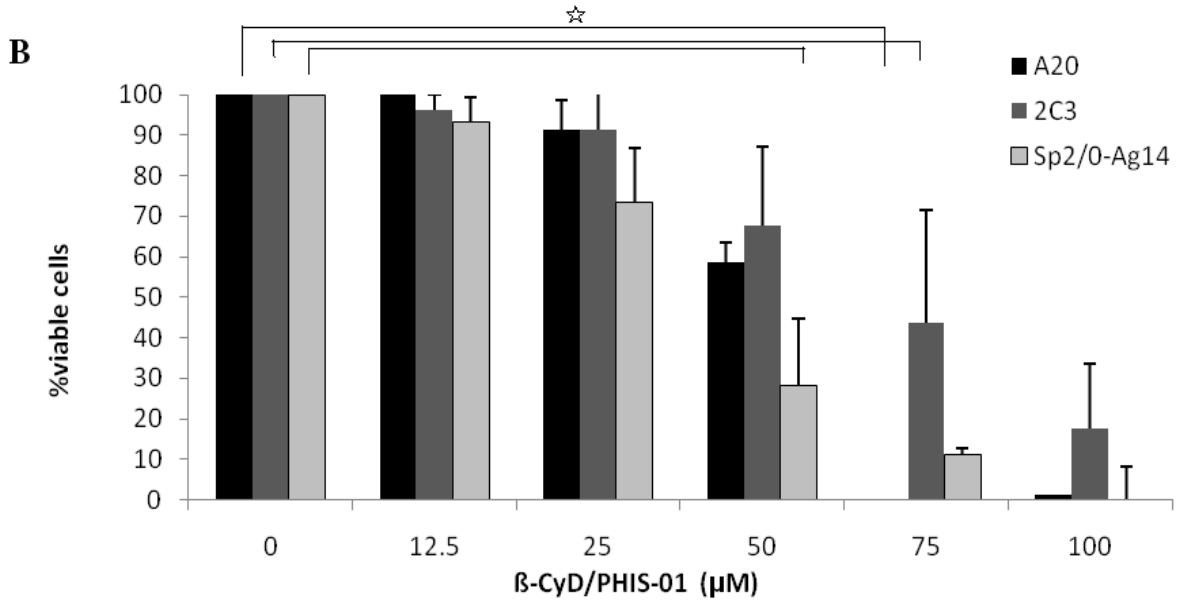


Figure 12.

Effects of Adjuvant-ß-CyD inclusion Complexes on Viability of Mouse Lymphoma Cells 2C3, A20, and SP2/0-Ag14.

Cells were treated for 24 hrs with various concentrations of different test agents, and then examined with MTS assay. The data are presented as the mean ±S.D. for three independent experiments, each in triplicates.

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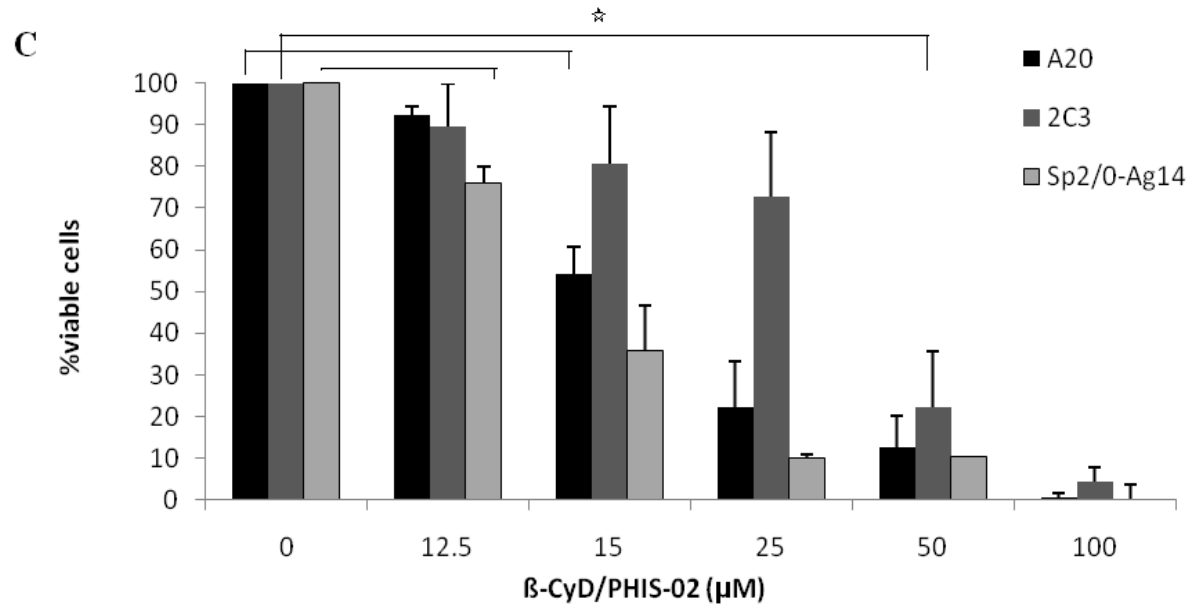
ß-CyD/PHIS-01 (µM)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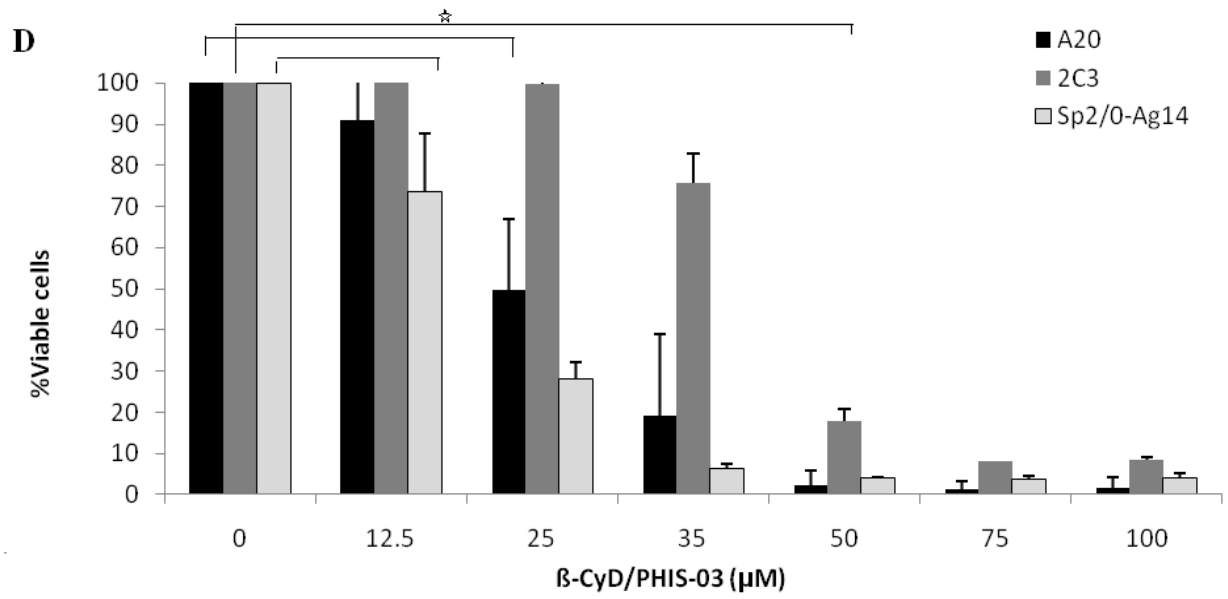


Figure 13.

Effect in Vitro of ß-CyD/Phytol, ß-CyD/PHIS-01, ß-CyD/PHIS-02 and ß-CyD/PHIS-03 on 2C3 Membrane Integrity.

Cells were treated for 24 hrs with various concentrations of different test agents, and then assessed by LDH release assay. The data are presented as the mean ±S.D. for three independent experiments, each in triplicate.

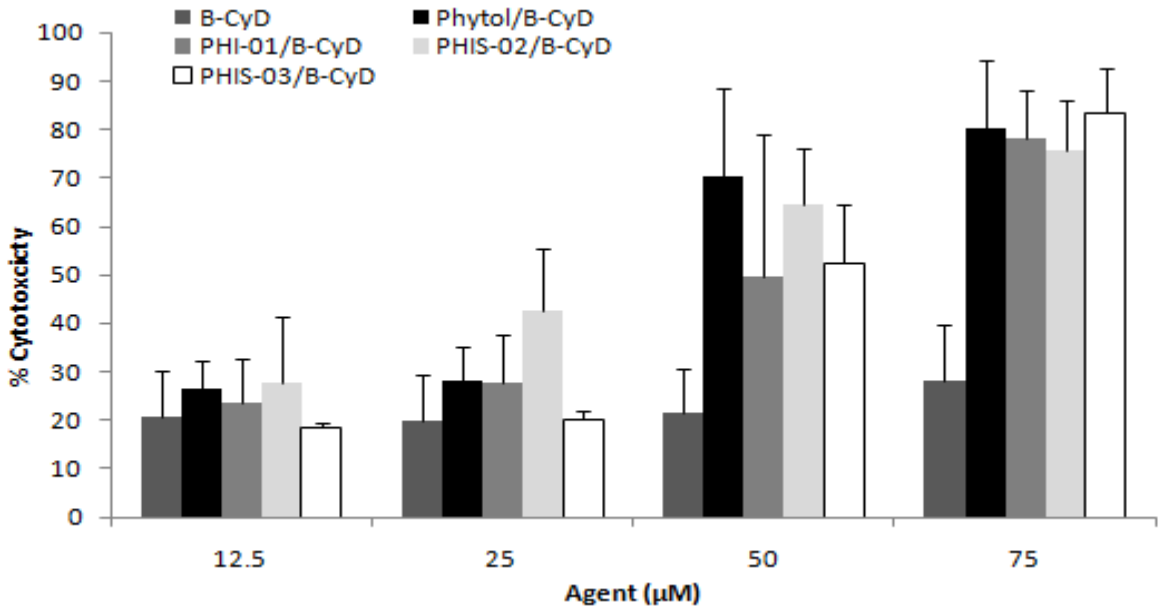


Figure 14.

Assessment of Cellular Apoptosis by DNA Fragmentation Assay in a Lymphoma Treated with Various Adjuvants.

2C3 tumor cells were treated for 24 hrs in the presence of ß-CyD alone (lane 2), 50 µM of ß-CyD/phytol (lane 3), 75 µM of ß-CyD /Phytol (lane 4), 50 µM of ß-CyD/PHIS-01 (lane 5), 75 µM of ß-CyD/PHIS-01 (lane 6), 50 µM of ß-CyD/PHIS-02 (lane7), 75 µM of ß-CyD/PHIS-02 (lane 8), 50 µM of ß-CyD/PHIS-03 (lane 9), 75 µM of ß-CyD/PHIS-03 (lane 10). As described in materials and methods, equal amounts of DNA isolated from all groups were elecrtophoresed in 2% agarose gels, and compared for evidence of DNA fragmentation.

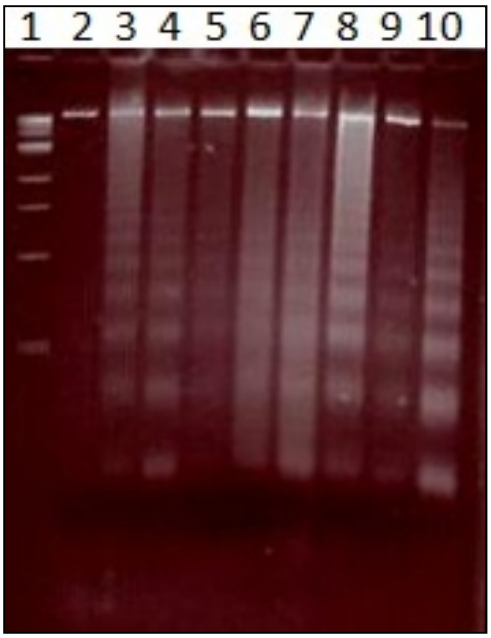
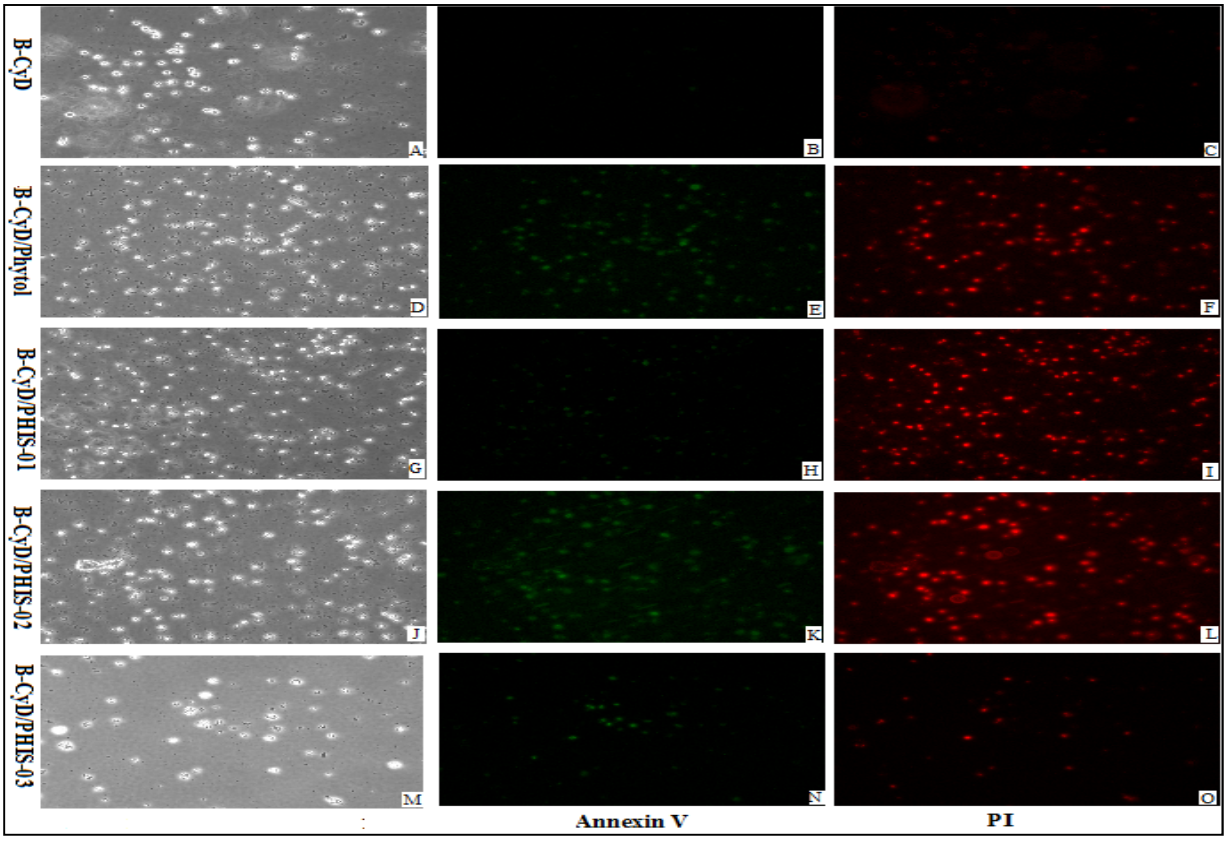


Figure 15.

Assessment of Apoptosis/necrosis.

Fluorescent microscopic examination of 2C3 cells treated with either ß-CyD/Phytol, ß-CyD/PHIS-01, ß-CyD/PHIS-02 or ß-CyD/PHIS-03, followed by staining with Annexin V or PI. 2C3 cells were treated for 24 hrs with either 50 µM of ß-CyD (A, B, C), ß-CyD/phytol (D, E, F), ß-CyD/PHIS-01 (G, H, I), ß-CyD/PHIS-02(J, K, L), and ß-CyD/PHIS-03(M, N, O). Transmission microscopic pictures are shown of treated and untreated samples labeled (A, D, G, J, and M).



CHAPTER 4

TOPIC 2: MOLECULAR SIGNATURES OF PHYTOL-DERIVED IMMUNOSTIMULANTS IN THE CONTEXT OF CHEMOKINE-CYTOKINE MICROENVIRONMENT AND ENHANCED IMMUNE RESPONSE

Abstract

In a previous report, we observed that the phytol-derived immunostimulant, PHIS-01 (phytanol), is a nontoxic oil-in-water adjuvant which is superior to most commercial adjuvants. In contrast, the parent diterpene alcohol phytol, though highly effective as an adjuvant, is relatively toxic. To assess the importance of the polar functional group in PHIS-01, we prepared two new compounds PHIS-02 (phytanyl amine) and PHIS-03 (phytanyl mannose). All three phytol derivatives proved to be excellent adjuvants, but differed in solubility and mode of action. To delineate their molecular signatures in the local microenvironment, we performed inflammasome and cytokine microarray analyses with the peritoneal fluid of mice treated with alum or the phytol compounds above, in the presence or absence of soluble protein antigens. We report here that the phytol derivatives had a significant time-dependent impact on the host chemokine-cytokine microenvironment and subsequently on specific humoral responses. Moreover, the inclusion of protein immunogens induced further changes in host microenvironments, including rapid (< 2 hr) expression of cytokines and chemotactic factors (IL-6, MCP-1, KC, MIP-1 and LIX), implying mobilization and activation of neutrophils, and

monocytes. PHIS-01 proved to be the most effective in this regard. Inflammatory cytokine cascades were dominant even after 24 hours possibly to facilitate involvement of the acquired immune system with the release of B-lymphocyte chemo-attractant BLC, T-cell activation-3 chemokines TCA, IL-4, IL-12, and TIMP-1. We also noted enhanced expression of NLRP genes especially NLRP3 with both alum and phytol derivatives (particularly PHIS-01).

Introduction

Safe vaccines are ideally constructed of non-replicating, and poorly immunogenic components of offending agents. Adjuvants improve immunogenicity by induction of significantly robust immune responses against poorly immunogenic substances. This productive and often protective immune response is characterized by mobilization and activation of innate immunity, elicitation of high-titer neutralizing antibodies and/or cell-mediated effectors that culminate in elimination of the offending agents. Adjuvants significantly impact both the quality and magnitude of the immune response. This is also evident in the generation of memory cells which respond quickly and efficiently during subsequent encounter with the same or cross-reactive offenders. However, there is no single mechanism to explain precisely how adjuvants mediate and augment immunogenicity. This is essentially because adjuvants are a chemically diverse group of compounds.

Only a few adjuvants are approved for use in human vaccines. These include alum (an inorganic salt) and MF59 (an organic oil-in-water emulsion). However, these may not be useful for every immunogen and adjuvants are often selected empirically. This is less than ideal and a rational approach based on adjuvant properties would be more desirable. This led us to search for common denominators such as cytokines or bio-response modifiers differentially induced by adjuvants in the presence of immunogens. It is important to point out

that both adjuvants and immunogens affect the cytokine microenvironment systemically and at the entry points. Vaccine efficacy is the net outcome from the combined contributions of both adjuvants and immunogens.

Adjuvants that are made up of microbial products elicit immune response through Toll-like receptors (TLRs) and Nod-like receptors (NLRs)[188-191]. Their interaction causes rapid mobilization and activation of the cells of innate immunity, such as dendritic cells, macrophages and monocytes, as a result of changes in host microenvironment with the release of cytokines and chemokines. The latter in turn orchestrates recruitment and interaction of cells belonging to both innate and acquired immunity [39, 140, 156, 192]. However, not all adjuvants are TLR-dependent. Alum, a hydrophilic salt, and oil-in water adjuvant MF59 based on squalene are TLR-independent. Adding to the complexity is that there are also regional differences, meaning that the routes of immunization may play important roles in the dynamics of microenvironments. At peritoneal sites in mice, alum causes an increase in monocyte chemotactic proteins (MCP1; CCL2), the neutrophil chemotaxin KC (CXCL1), and the eosinophil chemotaxin eotaxin-1 (CCL11) [39]. In contrast, MF59 has been shown to be a stronger inducer of cytokines [18, 156, 193].

Recently a number of studies have described how exposure to alum and MF59 causes distinctive changes in the molecular microenvironment in the host [18, 193]. Squalene (the organic component in MF59) is an endogenous triterpenoid metabolic product, whereas phytol-based adjuvants are exogenous diterpenoids, phytol being derived from chlorophyll in green plants. We have developed chemically modified phytol-based immunostimulants, including PHIS-01 (phytanol), PHIS-02 (phytanylamine), and PHIS-03 (phytanyl mannoside)[146-149]. These compounds exhibit excellent adjuvanticity, but differ significantly in the quality of

immune responses they engender. In some respects such as in the magnitude or fine specificity of the immune response, they appear to be superior to alum or Freunds' adjuvants [141, 142]. Alum promotes recruitment and migration of antigen presenting cells (APCs), induces necrosis in unidentified target cells resulting in the production of uric acid and stimulation of the NLRP3 inflammasome [39, 192]. Antibody response to alum-adsorbed ovalbumin (OVA) is abolished in NLRP3 knockout mice [192]. We have observed that phytol derivatives function by exerting apoptotic/ necrotic effects on target cells (manuscript submitted). It is possible that these effects facilitate recruitment of antigen-presenting cells (APCs) and promote cross-talk with the acquired immune system. However, with the phytol derivatives, these apoptotic/necrotic effects did not persist as happens with Fruend's adjuvants. We therefore hypothesize that phytol-based adjuvants are highly effective due to their ability to induce a nonpathogenic inflammatory reaction elaborating cytokines and chemokines capable of the recruitment and activation of APCs. In this study, we compared the immunocompetence of phytol-based adjuvants with alum in terms of effects on the cytokine/chemokine microenvironment of mouse peritoneal exudates (PE) at different time points after injection using microarray and RT PCR inflammasome array.

Our results indicate that within 2 hr post-injection, levels of cytokines and chemotactic factors (IL-6, MCP-1, KC, MIP-1 and LIX) are increased. The levels of these factors are more pronounced with PHIS-01 than PHIS-02 or PHIS-03. At 24 hours after injection, the inflammatory cascade leads to other cytokines, such as B-lymphocyte chemo-attractant B (BLC), IL-4, and IL-12 implying recruitment of the cells of acquired immunity. Like alum, phytol-based immunostimulants boost expression of the NLRP gene family, particularly NLRP3 and other inflammasome complexes, suggesting that PHIS-01 acts through activation of the NLRP3 Inflammasome. The functional moieties at the polar terminus of these phytanyl derivatives are important determinants of the nature of immune response and cytokine microenvironment they help induce.

Materials and Methods

Animals

Six-to eight weeks old female BALB/c mice used were housed and bred in the animal facility of Indiana State University according to the principles of laboratory animal care (NIH publications 85-23). This study was conducted using a protocol specifically approved by the ISU Institutional Animal Care and Use Committee (IACUC).

Chemicals and Reagents

Reagents used for in this study were purchased from the following sources: Alum (aluminum hydroxide gel), phytol (a racemic mixture of stereoisomers), Ovalbumin (OVA) and keyhole limpet hemocyanin (KLH) were obtained from Sigma-Aldrich Inc (USA). Other reagents included the Mouse Inflammasomes RT² Profiler™ PCR Array from SaBiosciences, a subsidiary of Qiagen Inc, USA, and the Cytokine-chemokine microarray from RayBiotech Inc.

Preparation of Adjuvants and Vaccine Formulation

Phytol-based immunostimulants PHIS-01, PHIS-02 and PHIS-03 were developed by chemical modifications of Phytol [146-149]. PHIS-01(phytanol) was prepared from phytol by reduction of phytol , and PHIS-02 (phytanyl amine) and PHIS-03 (phytanyl mannose) were derived from PHIS-01 according to published procedures[146-148]To assess changes in the host microenvironment due to phytol-based compounds in vaccine formulations, 8 to 10 week-old BALB/c mice were immunized intra-peritoneally with 100 μ g of OVA or KLH emulsified with either PHIS-01 (40 mg), PHIS-02 (2, 5 mg) or PHIS-03 (5 mg) in 500 μL of saline. The control groups consisted of (1) mice injected with adjuvants alone; (2) mice injected with 100 μ g of OVA or 100 ug of KLH alone; and (3) mice injected with saline alone. All experiments were performed and repeated with at least three BALB/c mice for each test group.

Collection of Peritoneal Cells and Lavages

BALB/c mice immunized as described above were sacrificed at 2 hr, 24 hr and 72 h after injection. PBS(3 mL) was used to harvest peritoneal lavages with 19 gauge needles, the collected samples were centrifuged (4°C, 400 g, 10 min), and supernatants used for cytokine and chemokine analysis. Peritoneal cells were washed twice with PBS and used for profiling inflammation-related gene expression.

Determination of Cytokines and Chemokines Secreted in the Peritonea

Cytokines and chemokines in peritoneal fluids were assessed using the mouse inflammatory Cytokine Array II (RayBiotech) following the manufacturer's instructions. Briefly, cytokine array membranes provided were blocked in 2 ml of blocking buffer for 30 min and then incubated with 1 mL of undiluted samples at 4^{o}C for overnight. Samples were then decanted, and the membranes washed three times with wash buffer. Membranes were

incubated with biotin-conjugated primary antibodies (1: 250 dilution) at room temperature for 2 hr, then washed and exposed to horseradish peroxidase-conjugated streptavidin (1:1000 dilution) for 1 hr. This was followed by treatment for 2 min with 500 µL of peroxidase substrate in the dark, and exposure of the membranes to X-ray film (Kodk X-OMAT AR film). Subsequently, the films were developed and signal intensities of all spots were analyzed to determine relative expression indices of cytokines released.

Cytokine Quantification

Cytokine signal intensities were quantified and analyzed with Image J software available from NCBI [152]. Positive controls and negative controls at six spots were used to normalize the results in different membranes. For each spot, the net optical density was determined by subtraction of background density from sample density and normalization to a positive control provided in each cytokine array. Results were expressed as relative intensity (RI) as percent of control.

Inflammasome-related Gene Expression by Quantitative RT-PCR Array

RNA was extracted from peritoneal cells using a commercial protocol (Ambion, Austin, TX). RNA quality was first assessed spectrophotometrically. All samples had 260/280 ratios above 2.0 and 230/260 ratios above 1.7. Further assessment was done using quality control plates (PAMM-999A-1, SA Biosciences, Frederick, MD). Then the RNA preparation was subjected to first-strand cDNA synthesis followed by PCR amplification. We used RT2 Profiler PCR inflammasome array (PAMM-097) and (SA Biosciencescatalogue # 330520). The experiments were performed in a Stratagene Mx3000P cycler. Five house-keeping genes, RT controls, and PCR controls were included. Data were analyzed and fold changes in values

calculated using PCR Array (SA Biosciences, http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php website), and expressed as average $\log_2$ ratios.

Results

Cytokine and Chemokine Protein Profiling in Mouse Peritoneal Fluids

Adjuvants such as the widely used hydrophilic aluminum hydroxide or aluminum sulfate hydrate (alum) have been shown to function by evoking changes in cytokine microenvironment at the vaccination site [18, 39, 192, 193]. We have developed hydrophobic phytol-derived immunostimulants, and examined effects of variation of structure at the polar terminus. In order to understand the molecular mechanisms underlying their adjuvanticity, we determined their effects on cytokine-chemokine at the peritoneal site of injection. Mice were immunized (I.P.) with alum, PHIS-01, PHIS-02 and PHIS-03 and changes in the profile at 2hr, and 24 hr were determined using commercial cytokine antibody arrays (Table 3a) and inflammasome-related gene expression arrays (Table 3b). Control mice treated with phosphate-buffered saline (PBS) served as the control. Since adjuvants primarily serve to boost the immunogenicity of antigenic components in a vaccine formulation, we compared the effects on cytokine microenvironments with and without immunogens OVA and KLH.

Mobilization of Innate Immunity by Adjuvants Alone

Chemotactic Factors in BALB/c Peritoneal Sites

We examined chemokine expression using commercial antibody arrays in which chemokine levels were semi-quantified and normalized with respect to positive controls provided with each test membrane (Figure 16). Analyses reveal that the PBS-treated control groups at 2 hr after injection showed a somewhat elevated level of KC (CXCL1), a neutrophil chemo-attractant, which declined within 24 hr (Figure 16A & 16B) . The constitutively

expressed macrophage inflammatory protein MIP-1γ (CCL9) remained steadily high in all groups including the PBS control. Adjuvants however made a much greater impact. The results in Figure 16A show that as early as 2 hrs after immunization, all adjuvanted groups seemed to respond similarly with high levels of pro-inflammatory chemokines KC (CXCL1), LIX (CXCL5), MCP-1(CCL2). While KC declined over 24 hr, both LIX and MCP-1 remained high (Figure 16B). Surges in these chemokines imply mobilization of neutrophils, eosinophils and monocytes [194]. All phytol derivatives were as effective as alum in this regard. Indeed, effects were more pronounced with PHIS-01. Additionally, the phytol-based adjuvants induced secretions of the chemokine BLC (CXCL13), that strongly and selectively attracts B lymphocytes, possibly B1 type in the peritoneum [195]. At 24 hr phytol compounds, but not alum, induced secretion of the growth factor G-CSF. Moreover, Eotaxin-2 (CCL11), a chemokine that mobilizes eosinophils trend up in all groups except in the PHIS-02 groups. In difference to PHIS-02, PHIS-03 and alum, PHIS-01 induced more RANTES, SDF-1, MIG, Fas ligand, and Fractalkine.

Cytokines in BALB/c Peritoneal Sites

The cytokine antibody array results (Figure 17A & 17B) indicate that within 2 hr after immunization, all adjuvant groups evoked high levels of the pro-inflammatory cytokine IL-6 which remained high even after 24 hrs in the phytol-based adjuvant groups but declined steeply in alum- and PBS-treated mice. In view of the cohort of chemokines (MCP-1, MIP-1α and eotaxin-2) that were released in response to alum and phytol compounds (Figure 16B) it is likely that the IL-6 elevation would largely be due to the recruitment of macrophages, monocytes and eosinophils. Cytokine microarray results (Figure 17B) also suggest that adjuvants PHIS-01 and PHIS-03 provoked a polarized T-lymphocyte response that developed

24 hr post-injection. In particular, PHIS-01 evoked more T-cell activating chemokines like RANTES (CCL5) and TCA3 as well as cytokines IL-12 and IL-4 than any other group.

Soluble TNF receptors I and II were secreted as early as 2hrs, with more sTNFR I produced than sTNFR II. The phytol-based adjuvants and alum were comparable in inducing sTNFR I/II secretion. Both phytol compounds and alum induced moderate levels of IL-1α, IL-4 and IL-12, 24 hr after stimulation. Immunization with PHIS-01 or alum induced detectable levels of IL-1β, IL-2, IL-13, IL-12 P70, and CD30 ligand. Only PHIS-01 induced TNF-α implying T helper type 1 response, while alum evoked detectable levels of IL-9 and IL10 indicating a T helper type 2 response. TIMP-1**,** a natural inhibitor of the matrix metalloproteinase, was moderately expressed after 2 hr but increased substantially after 24 hr following exposure to the adjuvants. Notably, PHIS-02 and PHIS-03 did not evoke any IL-2, and IL-13 even after 24 hr.

Antigen-mediated Modulation of the Cytokine and Chemokine Profile in the Presence or Absence of Adjuvants

To understand the dynamics of the cytokine microenvironment as influenced by both adjuvants and antigens, we assessed the cytokine milieu at peritoneal sites 24 hr after administration of soluble protein antigens KLH and OVA with or without alum or PHIS-01, PHIS-02, and PHIS-03. The data in Figures 18 and 19 showed that both antigens KLH and OVA were capable of inducing a large pool of cytokines and chemokines (notably, BLC, Eotaxin-2 , LIX, MCP-1, MIP-1γ,TCA3, M-CSF, IL4, IL12p40p70, IL-1α, sTNFRI, sTNFRII), albeit at varied levels. These chemokine/cytokine responses induced by OVA and KLH were qualitatively similar, but varied in details. The scenario changed when adjuvants were mixed with the antigens. OVA emulsified with PHIS-02 or PHIS-03 (but not PHIS-01)

reduced the expression levels of IL-6, MCP-1, G-CSF, sTNFRII as compared to adjuvants alone. In contrast, KLH with phytol-based adjuvants had no effect on the expression of these cytokines. All vaccine preparations containing adjuvantsand antigens moderately affected the levels of IL-12 P70P40, IL-α, IL-4, BLC, and M-CSF and lowered the expressions of TNF-α, IL-β, IL-2, IL-13, IL-9, IL-10, IL-12 P70, lymphotactin, TCA-3 and Rantes. Innocula containing PHIS-03 with KLH induced the highest level of eotoxin- 2, whereas OVA plus PHIS-01 evoked a higher level of BLC. Compared to alum, PHIS-01 influenced peritoneal cytokine/chemokine levels more profoundly, and PHIS-02 was similar to PHIS-01 though less robust.

To gain a better insight into the cytokine/chemokine molecular signature, we profiled 84 key gene expression levels using inflammasomes RT² Profiler™ PCR Array from SABiosciences with transcriptomes from peritoneal exudate cells (PECs) harvested 24 hr after administration of PHIS-01, PHIS-03 or alum. Since PHIS-01 and PHIS-02 largely evoked similar responses, this study was focused on PHIS-01, PHIS-03, and alum which served as the standard adjuvant for comparison. Analysis of Table 4 showed that PHIS-01 ,PHIS-03 and alum with or without OVA caused a marked up-regulation of MCP-1 related genes (MCP-3(CCL7), MCP-5(CCL12)) and MIP-2β (above 1.5 fold) in agreement with what we observed with cytokine antibody array described earlier. Gene expression of MIP-2β (CXCL3) was down regulated only in groups injected with Ova emulsified PHIS-03. KC and Rantes gene expression 24 hr after administration of PHIS-01 or alum was low and significantly down-regulated when PHIS-03 was used alone or in combination with ova.

Analysis of cytokines gene expressions (Table 5) showed that PHIS-01 alone or with OVA up-regulated expressions of INF-γ, IL-12b, IL-1β, IL-33, CD40L, TNF, TNFsf11,

TNFsf14 and TNFsf4. Alum with or without OVA up-regulated the expressions of INF-β, IL-1β, IL-12b, IL33, TNF, TNFsf14 and TNFsf11. On the other hand, PHIS-03 only up-regulated TNF, IL-1β,and IL-12b, and significantly down regulated IL-12a, IL-18, IL-33, CD40L gene expression. Interestingly, IL-6 gene expression was significantly down-regulated after 24 hr, whereas at the protein level, IL-6 expression remained high and steady as shown in Figure 16 and Figure 17. In a future study, we would attempt to resolve this contradiction.

Phytol-based Adjuvants Activate Several NLR Family Genes and Other Genes Involved in Formation of Different Inflammasome Platforms

PECs harvested 24 hr after treatment with PHIS-01, PHIS-03 or alum alone or as vaccine formulations with OVA were used to prepare total mRNA which was reverse transcribed to cDNA. The inflammasome pathway array was used to assess differential gene expressions in different experimental groups and compared with the PBS group. The relative gene expression was evaluated as fold increases. Of the 84 genes involved in the inflammasome pathway, pro-inflammatory cytokines and chemokines effectors genes were shown in Table 4& 5. Genes encoding the pattern recognition receptors (PRRs) family members of innate immunity, namely AIM2, NLRC4 or IPAF, NLRP1, and NLRP3 and other inflammasome-related components were shown in Table 6. Genes involved in downstream signaling during immune activation were grouped in Table 7, and a fold change ± 1.5 in gene expression relative to the PBS control was considered significant and noted.

Analysis of Table 3 shows that both alum and PHIS-01 up-regulated the expression of NLR gene; NLRP3, NLR4b, NLRP4e, NLRP5, NLRP6, NLRP9b, NLRP12, NLRX1and NOD 2, however, PHIS-03 only activated NLRP12 and NLRX1. All adjuvants down regulated genes involved in the Aim2 or IPAF inflammsomes (Aim2, Pycard (Asc), Nlrc4 (Ipaf), Naip1,

Naip5). Both alum and PHIS-01 but not PHIS-03 activated genes that have pro or anti-apoptotic activity such as Bcl2l1, Birc3, Cflar, and only activated caspase12 but not caspase 1 or 8. Thioredoxin (TRX)-interacting protein (Txnip), an essential protein for activation of NLRP3, was up-regulated significantly by both alum and PHIS-01. PHIS-01 activated XIAP, a gene involved in the formation of NLRP1 platform. PHIS-01 and PHIS-03 had moderate effects on Ctsb, a gene known as amyloid precursor protein and shown to participate in NLRP3 inflammasome activation (fold change between 1. 3 and 1.5). None of these adjuvants influenced purinergic receptor P2X, ligand-gated ion channel7 (P2rx7). Activation P2rx7 nuclear receptor by endogenous adjuvant ATP is reported to activate the NLRP3 inflammasome pathway. A gene, MEFV (Mediterranean fever), was highly expressed after the stimulation of peritoneal exudates with alum or PHIS-01. MEFV gene causes induction of pyrin, an important player in inflammatory response. All adjuvants induced ptgs2 (prostaglandin-endoperoxide synthase 2) a key enzyme involved in inflammatory response.

Table 7 shows genes involved in inflammasome downstream signaling. The gene expression profiles show that alum, PHIS-01 and PHIS-03 activated significantly MapK13 (p38), a kinase activated by inflammatory process and MyD88, a receptor-associated adaptor protein in antigen-presenting cells (APCs). Both alum and PHIS-01 did induce the gene expression of Irf3, a transcription factor for IFN-γ activation. Genes involved in transcription factor NF-kβ pathway were not highly activated as expected since they are probably activated early after stimulation. Table 7 shows that alum and PHIS-01 and to a lesser degree PHIS-03 activated NFKBIA, an inhibitor of the activity of dimeric NF-kβ/REL complexes, while PHIS-03 and alum with or without OVA activated RAGE, a pattern recognition receptor. In contrast, PHIS-01 alone significantly down-regulated the gene expressions of RELA (-14.2 fold) and

TIRAP (-10.3 fold); however PHIS-01 together with ova up-regulated the expression of these two genes by1.4 fold over PBS control.

Activation of inflammasome protein complex leads to up-regulation of pro-inflammatory cytokines such as IL-1β, IL-18, or IL-33. Table 5 shows a markedly increased expression of IL-1β when alum or PHIS-01 was used (> 3.5 fold). PHIS-03 induced a moderate gene expression of IL-1 β (1.8 fold). PHIS-01 and alum stimulated IL-33 gene expression but not IL-18.

Overall the picture that emerges from the inflammasome study is summarized as a Venn diagram (Figure 20 and 21)

Discussion

Isoprenoids, in particular diterpenes, have been shown to improve resistance to infection, by enhancing immune responses [11, 12, 17]. Phytol, a diterpene alcohol from chlorophyll, is a highly effective immunostimulant, but somewhat toxic in mouse models [138]. This led us to question whether the double bond in the phytol contributes to its toxicity. We have subsequently showed that hydrogenated phytol (PHIS-01) is a more efficient and nontoxic adjuvant. We then addressed the relevance of the polar alcoholic group of PHIS-01in adjuvanticity. This has been done by modifying the polar terminus of PHIS-01 with either a single amine group (PHIS-02) or a mannose moiety (PHIS-03). These phytol-based compounds are all excellent adjuvants at a much lower dose than phytol, and in some respects seem superior to Alum or Freunds' adjuvants [141, 142, 149, 196]

This study addressed four issues: (1) whether phytol-based immunostimulants affect bioresponse modifiers in the same way as alum does; (2) whether different protein antigens impact bioresponse modifiers differently; (3) whether the antigens and adjuvants together as in

vaccine formulations evoke the same or selectively magnify the effects on cytokine milieu; and (4) whether phytol compounds involve inflammasome pathway as their primary modes of action. All phytol compounds we studied are in the category of oil-in-water adjuvants. There are only a few oil-in-water adjuvants licensed for human use, such as a triterpene squalene, a metabolic product. Considerable information on molecular signatures of squalene adjuvant compared to alum's has recently been available [8].

Our Cytokine/chemokine protein array data in this study show that phytol-based adjuvants (PHIS-01, PHIS-02 and PHIS-03) activate genes related to chemoattractants, factors that mobilize innate immune cells. Upon immunization, all phytol-based adjuvants provoke induction of neutrophil-activating chemokines KC (CXCL1) and LIX (CXCL5) and detectable expressions of RANTES (CCL5) and Eotaxin-2 (CCL11). We also observed high expressions of MCP-1 (CCL2) that is known to recruit and activate monocytes [197, 198]. In addition, phytol compounds evoked growth factors M-CSF, GM-CSF and G-CSF which trigger differentiation of monocytes into macrophages, dendritic cells, and stimulate granulocytes respectively [199-201]. Other chemotactic factors released in response to phytol -based adjuvants are chemo-attractants such as BLC (CXCL13) for B lymphocytes, MIP-1α (CCL-3) and MIP-1γ (CCL9) secreted by macrophages for granulocytes and DCs, and TCA and lympotactin for activated T cells and NK cells. Inflammasome-related microarray data provide further confirmation of chemokine/ cytokine gene up-regulation mediated by PHIS-01, and PHIS-03. Together these results indicate that the phytol compounds initiate and activate both the innate and acquired immunity by cell recruitment, increased endocytosis in monocytes, stimulation of monocytes differentiation into macrophage or DC, and activation of T cells. Phytol-based adjuvants induce IL-6, IL-1, TNF- α, as well as Pgst2, a key enzyme in

prostaglandin E2 (PGE2) synthesis. Pro-inflammatory cytokines like IL-6 are known to drive monocyte-differentiation toward macrophages at the expense of DC development [202]. Monocytes, that are under the influence of adjuvants differentiate into macrophages, respond strongly to pro-inflammatory stimuli and release cytokines like IL-1, IL-6, TNF-α, and IL-12 [203-205]. However it has been shown that presence of an inflammatory cytokine mix (TNF-α, IL-6, IL-1, PGE2) leads monocyte-differentiation all the way into mature DCs[206]. In contrast, alum is not only effective in evoking a wide range of cytokine/ chemokine-related transcriptomes like pro-inflammatory MCP-1, MIP-1, IL-1, IL-4, Rantes, Eotaxin 2, but also IL-6. Moreover, alum seems to act mainly on monocytes and macrophages but not on granulocytes. This is evident from the fact that alum evokes little G-SCF, a growth factor promoting granulocytes. These results are in conformity with the data from cytokine /chemokine protein analysis from previous reports [18, 39, 156, 207].

In this study we have also assessed the influence of antigens on innate immunity. Our results indicate that ovalbumin but not KLH down-regulate inflammatory chemokines (MCP-1, IL-6, and G-CSF) that are activated by PHIS-02, PHIS-03 and alum, but not PHIS-01. This agrees with our pervious findings which show that anti-phthalate-KLH antibody response was equally high with alum as well as phytol compounds as adjuvants. However, for induction of anti-OVA antibody, PHIS-01 proves to be the effective immunostimulant. It is important to note that different immunogens like OVA and KLH impact the microenvironment without the adjuvant by eliciting signature cytokines and chemokines. This is an intrinsic nature of immunogens that gets modulated again with the introduction of adjuvants. Overall impacts of vaccine are due to these combined effects. Studies evaluating just adjuvants do not often take

into account the role antigens also play in the cytokine milieu that would influence the ultimate immune response.

Phytol derivatives PHIS-01, PHIS-02 and PHIS-03 also activate innate immunity at injection sites as do alum and MF59 of squalene [18, 193, 207], The core response genes up-regulated by phytol derivatives include not only cytokine and chemokines but also Nod-like receptor genes and associated molecules which reportedly activate or participate in the formation of inflammasome platform . This suggests that phytol derivatives like alum mediate their effects through inflammatory pathway related to inflammasome. The importance of NLRPs or other molecules involved in inflammasome pathways in vaccine adjuvanticity is well documented.

Here we show that PHIS-01 works in a manner similar to alum by up-regulating the expressions of NLRP-3, Mefv, CtsB and Txnip. The products of these genes are essential for NLRP3inflammsome formation and activation [72, 208-211]. Furthermore, we observed downstream modulation of the transcription factor NF-kβ, the enzyme for cell proliferation MapK, and interferon-regulatory factor IRF. These molecules are involved in proinflammatory cytokines secretion and regulation. Additionally, there is a significant increase in IL-1β both at the mRNA level and at corresponding protein level, but this is observed only with PHIS-01 and alum, but not with PHIS-02 or PHIS-03.

The difference among the phytol adjuvants at the cytokines and chemokines levels is more quantitative than qualitative. PHIS -01 was the most potent inducer of chemokines and cytokine compared to PHIS-02 than PHIS-03. PHIS-01 and PHIS-02 are similar in their effects as adjuvants, PHIS-03 is however different. At the levels of gene expression, PHIS-01 is more potent than PHIS-03 in the induction of a plethora of Nod-like receptors, cytokine/ chemokines

and downstream signaling molecules. This finding may account for the ability of PHIS-01 to activate Th1 and Th2 responses; in contrast PHIS-03 only activated Th2 response. It is apparent that innate immunity plays an important role in the adjuvanticity of phytol derivatives.

The engagement of the innate immunity seems to occur due to apoptotic and/or necrotic effects induced by phytol compounds at the site of injection, as has been observed with many necrosis-inducing stimuli [212]. However, both PHIS-01 and PHIS-03 also activate a cluster of molecules such as IL-1α, Timp-1, Cflar, Bir 3, and XIAP that function as tissue injury response molecules or for blocking of apoptosis. Again, PHIS-01 was a very potent inducer of these genes which implies a higher apoptotic/necrotic activity; as a consequence there is a marked release of danger signal molecules from the surrounding tissues and that would explain the upregulation of several Nod-like receptors and subsequent activation of innate immunity. Interestingly, this controlled inflammatory response is well characterized by increased expressions of caspase12[213], NLRP-12 [214, 215], and MEFV [216, 217], a group of genes that control and prevent unfettered inflammatory response . This implies that the phytol derivatives improve vaccine immunogenicity by promoting regulated and nonpathogenic inflammatory changes in the immediate microenvironments.

Our finding also clearly highlights the importance of bonds and functional moieties in shaping the adjuvanticity of terpenoids like phytol derivatives. A simple hydrogenation of double bonds in phytol generates PHIS-01 which is a very safe and superior adjuvant in terms of the quality and magnitude of overall immune response evoked. In addition, modifications of polar terminus of PHIS-01 with a hydrophilic mannose moiety (PHIS-03) change the cytokine/chemokine profile, and this evidently leads to T helper polarization and magnitude of

antibody response. Although phytol compounds we tested are broadly effective, our ongoing study also suggests that they are ameliorative in autoimmune-prone NZB/WF1 mice.

Table 3a.

Commercial Proinflammatory Cytokine and Chemokine Array Template.

POS	POS	NEG	NEG	Blank	BLC	CD30L	Eoatxin	Eoatxin-2	FAS L	Fracatlkine	GCSF
POS	POS	NEG	NEG	Blank	BLC	CD30L	Eoatxin	Eoatxin-2	FAS L	Fracatlkine	GCSF
GM-CSF	IFN-γ	Il-1α	IL-1β	Il-2	IL-3	IL-4	IL-6	IL-9	IL-10	IL-12p40P70	Il-12P70
GM-CSF	IFN-γ	Il-1α	IL-1β	Il-2	IL-3	IL-4	IL-6	IL-9	IL-10	IL-12p40P70	Il-12P70
IL-13	Il-17	I-Tac	KC	Leptin	LIX	Lymphotactin	MCP-1	MCSF	MIG	MIP-1α	MIP-1γ
IL-13	Il-17	I-Tac	KC	Leptin	LIX	Lymphotactin	MCP-1	MCSF	MIG	MIP-1α	MIP-1γ
RANTES	SDF-1	TCA-3	TECK	TIMP-1	TIMP-2	TNF-α	sTNF R I	sTNF R II	Blank	Blank	POS
RANTES	SDF-1	TCA-3	TECK	TIMP-1	TIMP-2	TNF-α	sTNF R I	sTNF R II	Blank	Blank	POS

BLC, B lymphocyte chemoattractant; MCP, monocyte chemotactic protein; KC, [keratinocyte chemoattractant; eoatxin, eosinophil chemotactic protein; LIX, Neutrophil-activating protein; MIP, macrophage inflammatory protein; RANTES, Regulated upon Activation, Normal T-cell Expressed, and Secreted ; MIG, Monokine induced by gamma interferon; GM-CSF , Granulocyte-macrophage colony-stimulating factor G-CSF , Granulocyte colony-stimulating factor M-CSF, Macrophage colony-stimulating factor; SDF , stromal cell-derived factor-1; TIMP, tissue inhibitors of matrix metalloproteinases; TNF, Tumor necrosis factor; soluble Tumor necrosis factor receptor; TCA-3, T-cell activation-3; I-TAC, Interferon-inducible T-cell alpha chemoattractant; POS, Positive ; NEG, negative.

Table 3b.

Inflammasome-related Gene Expression Array Template.

Aim2	Bcl2	Bcl2l1	Birc2	Birc3	Card6	Casp1	Casp12	Casp8	Ccl12	Ccl5	Ccl7
Cd40lg	Cflar	Chuck	Ciita	Ctsb	Cxcl1	Cxcl3	Fadd	Hsp90aa1	Hsp90ab1	Hsp90b1	Ifnb1
Ifng	Ikbkb	Ikbkg	Il12a	Il12b	Il18	Il1b	Il33	Il6	Irak1	Irf1	Irf2
Irf3	Map3k7	Map3k7ip1	Map3k7ip2	Mapk1	Mapk11	Mapk12	Mapk13	Mapk3	Mapk8	Mapk9	mefv
Myd88	Naip1	Naip5	Nfkb	Nfkbia	Nfkbib	Nlrc4	Nlrc5	Nlrp12	Nlrp1a	Nlrp3	Nlrp4b
Nlrp4e	Nlrp5	Nlrp6	Nlrp9b	Nlrx1	Nod2	P2rx7	Panx1	Pea15a	Pstpip	Ptgs2	Pycard
Rage	Rela	Ripk2	Sugt1	Tirap	Tnf	Tnsf11	Tnfsf14	Tnfsf4	Traf6	Txnip	Xiap
Gusb	Hprt1	Hsp90ab1	Gapdh	Actb	MGDC	RTC	RTC	RTC	PPC	PPC	PPC

Table 4.

Transcription of Chemokine Genes in the Mouse Peritoneum.

The level of expression is shown as ± Fold change compared with PBS controls sacrificed at the same time point.

	OVA	ALUM	ALUM+OVA	PHIS-01	PHIS-01+OVA	PHIS-03	PHIS-03+OVA
MCP-5	1.0	2.8	1.6	2.2	2.4	2.0	1.3
RANTES	-1.8	1.1	1.1	1.4	1.1	-1.4	1.0
MCP-3	1.0	3.6	3.1	3.6	3.2	2.4	1.4
KC	1.3	1.3	1.2	1.0	-1.1	-1.5	-2.5
MIP-2β	-1.3	3.5	2.0	3.5	3.4	1.9	-1.3

Table5.

Transcription of Cytokine Genes in the Mouse Peritoneum.

The level of expression is described as ± Fold change compared with PBS controls sacrificed at the same time point.

gene	OVA	ALUM	ALUM+OVA	PHIS-01	PHIS-01+OVA	PHIS-03	PHIS-03+OVA
IFN-β1	1.2	1.9	3.3	1.2	-1.3	-1.3	-1.6
IFN-γ	-2.0	1.4	2.3	2.2	1.8	-1.4	1.3
IL-12a	-1.1	1.1	1.3	-1.0	-1.2	-1.9	-1.6
IL-12b	-1.2	3.4	5.0	3.6	3.0	1.7	1.9
IL-18	1.1	-1.2	1.1	1.1	-1.0	-1.6	-1.3
IL-1β	-1.3	4.4	3.8	4.2	3.1	1.8	1.2
IL-33	1.1	1.6	2.5	1.5	1.6	-2.0	-1.3
IL-6	-1.1	-1.4	-1.2	-1.6	-1.7	-2.6	-1.4
Cd40lg	-1.2	-1.3	1.4	1.5	1.2	-1.6	1.1
TNF	1.2	3.8	3.0	2.7	2.6	2.3	1.2
TNFsf11	-1.2	-1.1	2.5	-2.1	1.9	1.1	1.2
TNFsf14	-2.0	1.9	2.6	2.6	2.3	1.2	1.2
TNFsf4	1.5	1.0	1.6	1.5	1.7	-1.3	-1.1

Table 6.

Transcription of NOD-like Receptors and Inflammasome-pathway Associated Genes in the Mouse Peritoneum.

The level of expression is shown as ± Fold change compared with PBS controls sacrificed at the same time point.

Gene	OVA	ALUM	ALUM+OVA	PHIS-01	PHIS-01+OVA	PHIS-03	PHIS-03+OVA
Aim2	-1.3	-1.8	-1.1	-1.1	-1.3	-1.7	-1.3
Bcl2	-1.3	-2.3	-1.6	-1.2	-1.3	-1.7	-1.3
Bcl2l1	1.0	1.6	1.3	1.7	1.4	1.3	-1.1
Birc2	-1.0	-1.2	1.0	1.2	1.1	-1.6	-1.1
Birc3	-1.0	1.4	1.4	1.6	1.4	-1.2	-1.1
Card6	-1.4	-1.2	-1.1	1.3	1.1	-1.8	-1.5
Casp1	-1.0	1.0	1.1	1.1	-1.0	-1.1	-1.1
Casp12	1.4	1.3	3.3	1.4	-1.0	-1.1	-1.0
Casp8	-1.3	-1.9	-1.2	1.0	-1.2	-1.8	-1.2
Cflar	-1.1	1.4	1.4	1.6	1.5	1.0	1.1
Ctsb	1.2	1.3	1.5	1.3	1.3	1.3	1.1
Fadd	-1.1	-1.4	1.2	-1.1	-1.2	-2.1	-1.3
Naip1	-1.3	-2.8	-1.5	-2.1	-1.6	-2.2	-2.2
Naip5	-1.2	-1.7	-1.1	-1.1	-1.1	-1.5	1.1
Nlrc4	-1.3	-2.0	-1.5	-8.6	-1.3	-1.7	-1.3
Nlrc5	-1.4	-1.4	-1.2	1.1	1.2	-1.5	1.0
Nlrp12	-4.3	1.8	2.0	3.0	2.7	1.1	1.4

Table 6 (Continued).

Gene	OVA	ALUM	ALUM+OVA	PHIS-01	PHIS-01+OVA	PHIS-03	PHIS-03+OVA
Nlrp1a	-1.1	-1.2	1.3	1.5	1.6	-1.2	1.2
Nlrp3	-1.4	1.6	1.8	1.9	1.7	1.1	-1.0
Nlrp4b	-1.1	1.0	3.6	2.0	-1.3	-1.4	-1.2
Nlrp4e	-1.1	-1.1	2.4	1.6	-1.6	-1.6	-1.8
Nlrp5	-1.1	1.2	2.9	-1.2	-1.1	-1.4	-1.5
Nlrp6	1.2	-1.2	2.8	1.7	1.1	-1.9	-1.5
Nlrp9b	1.3	2.1	4.3	2.8	1.1	1.0	-1.1
Nlrx1	1.1	1.5	1.5	2.0	2.1	1.1	1.6
Nod2	-1.2	1.5	1.4	1.4	1.6	1.1	1.1
P2rx7	-1.4	-1.1	-1.1	-1.2	1.3	1.1	1.3
Panx1	1.0	-1.3	-1.0	-2.0	1.1	-1.1	1.1
Pea15a	1.1	-1.6	-1.0	1.2	1.2	1.1	1.2
Pstpip1	-1.1	1.7	1.6	2.1	1.9	1.5	1.3
Ptgs2	-1.1	3.6	3.8	5.6	4.4	1.5	1.1
Pycard	-1.1	-1.3	-1.1	1.3	1.2	-1.5	1.0
Txnip	1.0	1.5	1.7	1.6	1.5	1.1	1.1
Xiap	-1.0	1.1	1.2	1.5	1.4	1.0	1.1
Mefv	-1.1	3.3	2.7	3.3	2.7	1.2	-1.0

Table 7.

Transcription of Downstream Signaling Gene Involved in Inflammasome-pathway in the Mouse Peritoneum.

The level of expression is shown as ± Fold change compared with PBS controls sacrificed at the same time point.

Gene	OVA	ALUM	ALUM+OVA	PHIS-01	PHIS-01+OVA	PHIS-03	PHIS-03+OVA
Chuk	-1.2	-1.6	1.0	1.2	-1.0	-1.2	-1.1
Ciita	1.1	1.0	1.2	1.3	1.2	-1.4	-1.2
Ikbkb	-1.0	-1.4	-1.1	1.2	-1.0	-1.6	-1.1
Ikbkg	1.1	1.2	1.3	1.4	1.3	-1.1	1.1
Irak1	1.0	-1.5	-1.2	1.0	-1.1	-1.4	-1.1
Irf1	-1.0	1.6	1.4	1.7	1.4	-1.0	-1.0
Irf2	-1.1	-1.1	1.0	1.1	1.0	-1.1	-1.0
Irf3	-1.2	-1.3	1.3	1.4	1.2	-1.6	-1.0
Map3k7	-1.0	-1.4	-1.1	1.2	1.1	-1.5	1.0
Map3k7ip1	1.0	-1.1	1.2	1.2	1.2	-1.3	1.0
Map3k7ip2	-1.2	1.0	1.1	1.4	1.2	-1.2	1.0
Mapk1	-1.1	-1.2	-1.1	1.1	1.0	-1.2	1.0
Mapk11	-1.1	-1.9	-1.4	-1.1	-1.0	-1.5	-1.4
Mapk12	-1.0	-2.0	-1.1	-1.4	-1.3	-2.1	-1.5
Mapk13	-1.8	1.9	1.8	1.9	1.7	1.1	-1.2
Mapk3	1.0	1.5	1.3	1.4	1.3	1.1	1.3
Mapk8	-1.2	-1.6	1.1	1.1	-1.0	-1.4	1.0

Table 7 (Continued).

Gene	OVA	ALUM	ALUM+OVA	PHIS-01	PHIS-01+OVA	PHIS-03	PHIS-03+OVA
Mapk9	-1.1	-1.4	1.1	-1.0	-1.0	-1.6	-1.1
Myd88	1.2	1.8	1.4	2.4	2.1	1.7	1.2
Nfkb1	-1.1	-1.0	-1.1	1.3	1.1	-1.3	-1.0
Nfkbia	-1.1	1.6	1.3	1.5	1.3	1.1	-1.3
Nfkbib	-1.1	-1.0	1.4	1.2	1.4	-1.9	-1.1
Rage	-1.8	-1.8	1.5	1.2	-1.1	-1.6	-1.1
Rela	-1.0	1.2	1.1	-14.2	1.4	-1.1	1.1
Ripk2	-1.1	-1.4	-1.2	-1.2	-1.2	-1.3	-1.2
Tirap	-1.2	1.0	1.3	-10.3	1.4	-1.4	1.1
Traf6	-1.1	-1.2	1.1	-1.2	1.2	-1.2	-1.1

Figure 16.

Chemokine Profile Induced by Phytol-derivatived Adjuvants.

BALB/c mice were injected i.p. with either PHIS-01, PHIS-02, or PHIS-03, and peritoneal exudates harvested after 2hr (A), or after 24 hr (B). Control groups were treated with either the standard adjuvant alum or PBS buffer. Peritoneal fluids were assayed to determine chemokine expression as detailed in materials and methods. Data are expressed as the mean relative intensity relative to positive control of each chemokine protein detected using pooled peritoneal fluids of 3 mice per group in duplicate. The result is average of two separate experiments.

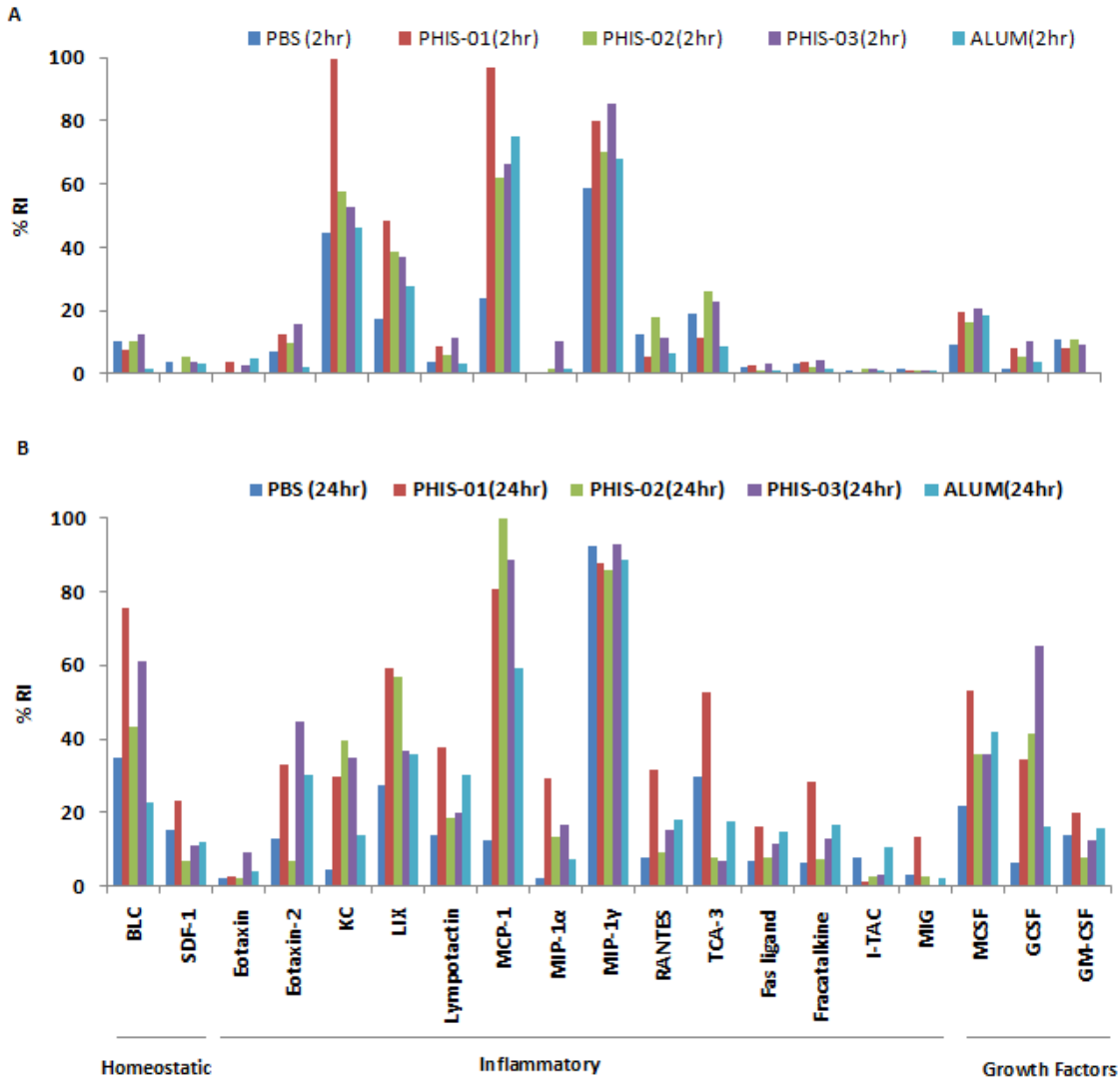


Figure 17.

Cytokine Profile Induced by Phytol-derivatives Adjuvants

BALB/c mice were injected i.p. with either PHIS-01, PHIS-02, or PHIS-03, and peritoneal exudates harvested after 2hr (A), or after 24 hr (B). Control groups were treated with either the standard adjuvant alum or PBS buffer. Peritoneal fluids were assayed to determine cytokine expression as detailed in materials and methods. Data are expressed as the mean relative intensity relative to positive control of each chemokine protein detected using pooled peritoneal fluids of 3 mice per group in duplicate. The result is average of two separate experiments.

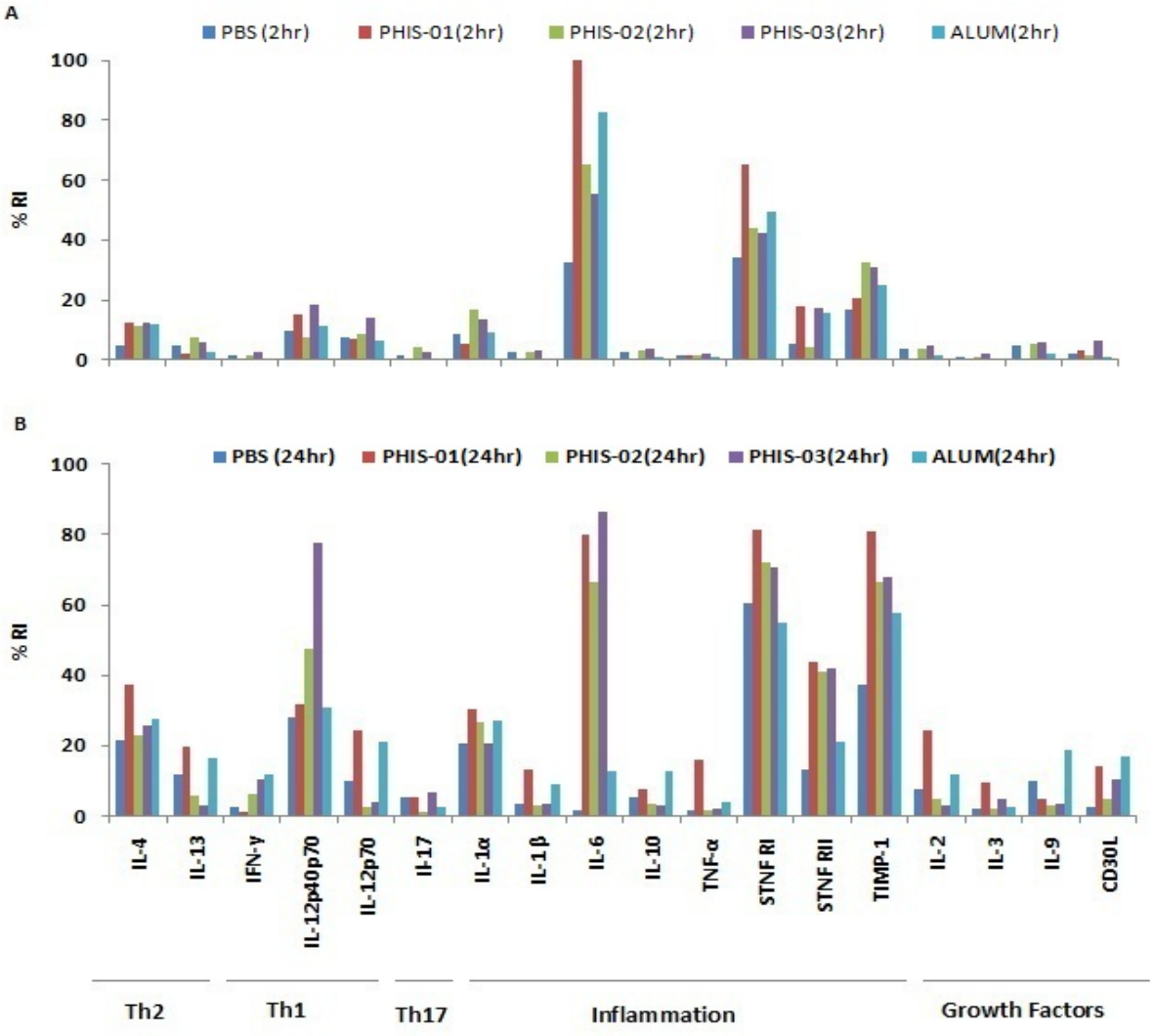


Figure 18.

Chemokine Profile Induced by Phytol-derivative Adjuvants in Inoculums with OVA or KLH. BALB/c mice were injected i.p. with OVA (A) or KLH (B) and either PHIS-01, PHIS-02, PHIS-03, or alum. Peritoneal exudates were harvested after 24 hr. The control group was treated with antigen alone. Peritoneal fluids were assayed to determine chemokine expression as detailed in materials and methods. Data are expressed as the mean relative intensity relative to positive control of each chemokine protein detected using pooled peritoneal fluids of 3 mice per group in duplicate. The result is average of two separate experiments.

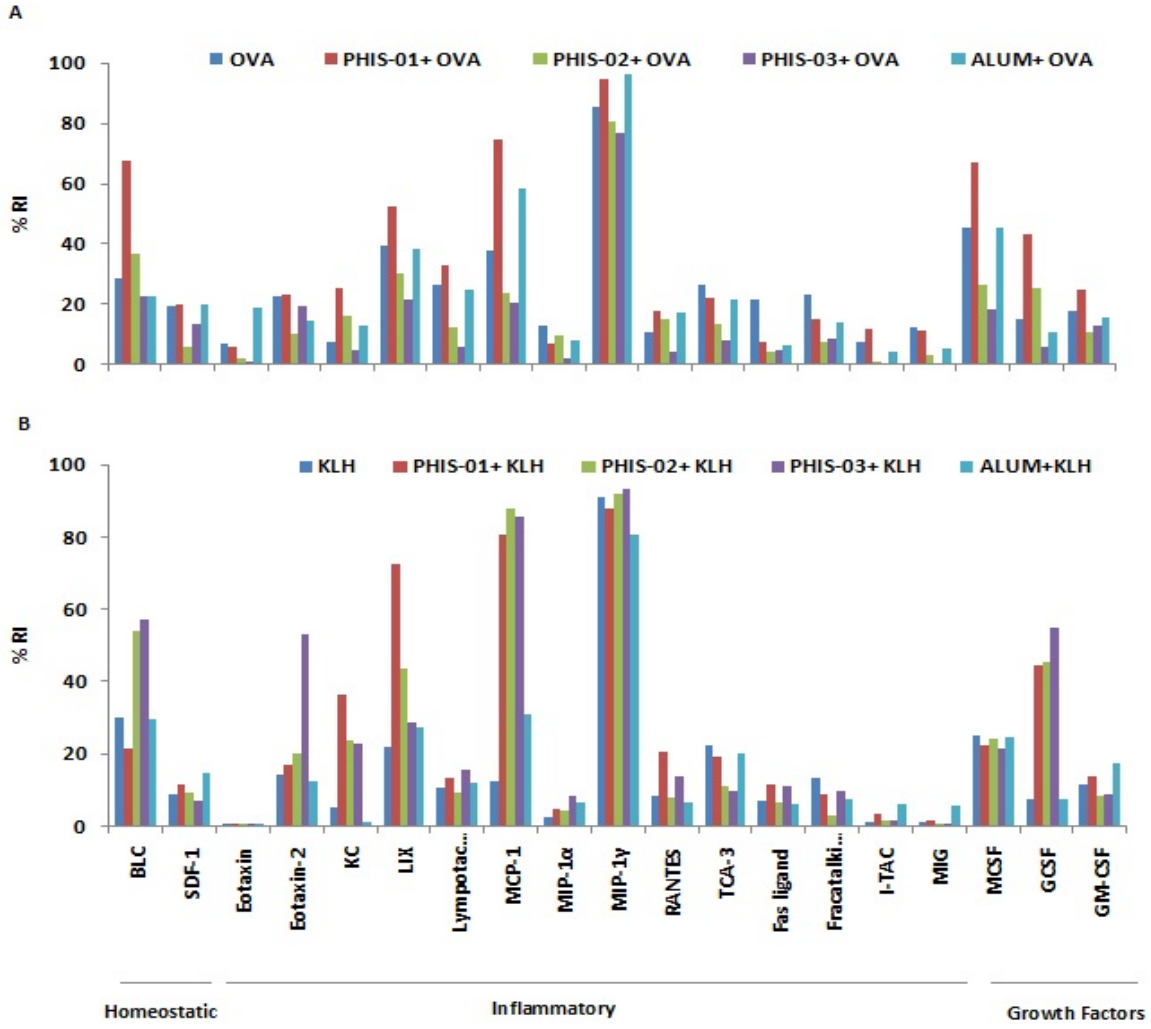


Figure 19.

Cytokine Profile Induced by Phytol-derivative Adjuvants in Inoculums with OVA or KLH. BALB/c mice were injected i.p. with OVA (A) or KLH (B) and either PHIS-01, PHIS-02, PHIS-03, or alum. Peritoneal exudates were harvested after 24 hr. The control group was treated with antigen alone. Peritoneal fluids were assayed to determine cytokine expression as detailed in materials and methods. Data are expressed as the mean relative intensity relative to positive control of each cytokine protein detected using pooled peritoneal fluids of 3 mice per group in duplicate. The result is average of two separate experiments.

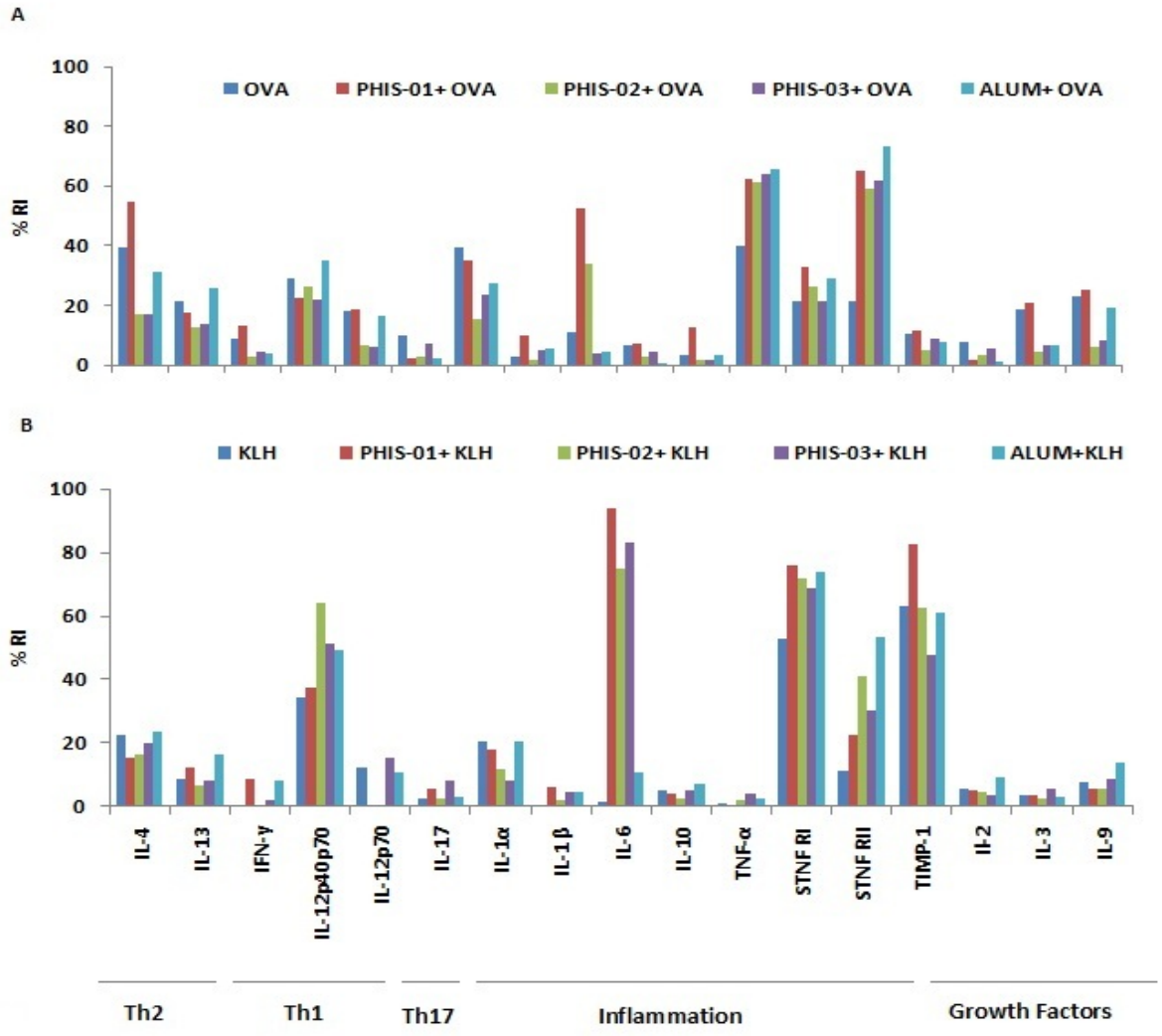


Figure 20.

RT-PCR Microarray Analysis of Transcriptome Profiles of Inflammatory Genes Induced by Vaccine Adjuvants alone in Mouse Peritoneum.

Genes (84) were assessed and those genes up-regulated (A), or down regulated with an average $\log_2$ ratio ≥ 1.5 were selected and plotted as Venn diagram

A

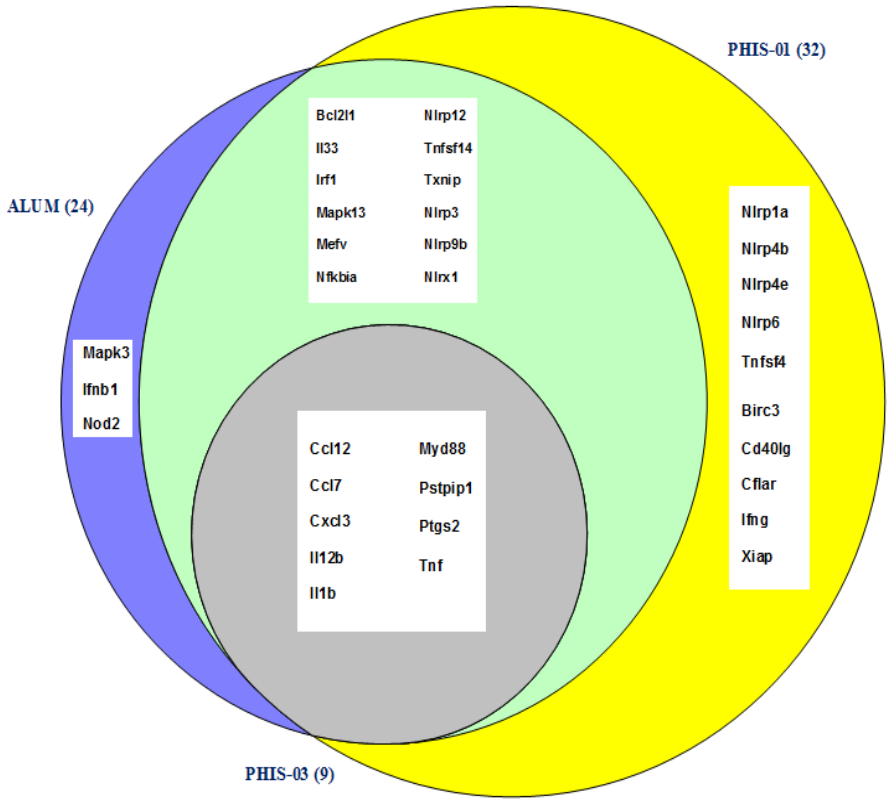


Figure 20 (Continued).

B

Figure 21.

RT-PCR Microarray Analysis of Transcriptome Profiles of Inflammatory Gene Induced by Vaccine Adjuvants in Combination with OVA in Mouse Peritoneum.

Genes (84) have been tested and only gene up-regulated (A), or down regulated (B) with an average log2 ratio ≥ 1.5 were selected and plotted as Venn diagram

A

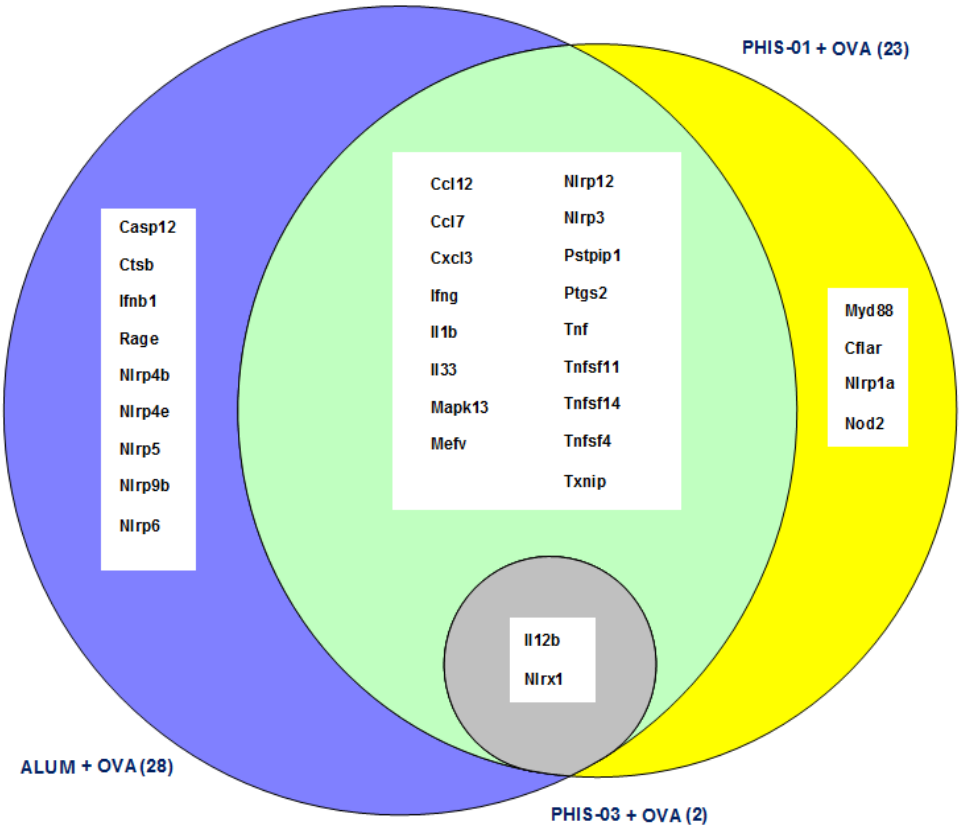


Figure 21 (Continued).

B

CHAPTER 5

TOPIC 3: EXTRACELLULAR MATRIX FROM PORCINE SMALL INTESTINAL SUBMUCOSA AS IMMUNE ADJUVANTS

Abstract

Porcine small intestinal submucosa (SIS) is widely used in tissue remodeling. This extra cellular matrix from Cook Biotech has also been used as an effective adjuvant in a prostate cancer vaccine model. The present study addressed whether SIS would be as effective as alum in a broader context in recruiting innate immunity via inflammasomes, and in boosting antibody responses to soluble proteins and hapten-protein conjugates. We used ovalbumin (OVA), and a hapten-protein conjugate, phthalate-keyhole limpet hemocyanin (KLH). The evaluation of SIS was conducted in BALB/c and C57BL/6 mice using both intraperitoneal and subcutaneous routes. Inflammatory responses were studied by microarray profiling of chemokines and cytokines and by qPCR of inflammasomes-related genes. Results demonstrated that SIS provoked neither pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) nor NLRP3 inflammasomes, but it did up-regulate IL4 and CD30-ligand. SIS activated chemotactic factors LIX and KC (neutrophil chemotactic factors), MCP 1 (monocyte chemotactic factors), MCSF and MIP 1-α (macrophage chemotactic factor) and Lympotactin, albeit a little less than Alum. Nevertheless, SIS was as effective as alum in engendering a lasting and specific antibody response, primarily of IgG1 type, but it did evoke low level of

anti-DNA response. Thus, although both alum and SIS induced Th2 type immune response, SIS did not function through NLRP3 inflammasomes.

Introduction

Ninety years have passed since the concept of adjuvants took hold in vaccine design, and they no longer regarded as "immunologists' dirty tricks". Thousands of chemicals have been assessed for their ability to enhance specific immune response; but the search is still on for the broadly effective adjuvants. Ideally, a versatile adjuvant should be safe but still be capable of engendering robust immune responses to a wide variety of offenders. It is not easy however to produce an ideal, broadly effective adjuvant from a single compound. Vaccine efficacy does not depend merely on adjuvants, but more importantly on the nature of the offenders that serve as immunogens. Adjuvants and immunogens together influence host immune microenvironment, and thereby modulate immunogenicity of a wide array of vaccines. However, no two adjuvants or immunogens interact in the same way, and effects of adjuvants are also subject to modifications by the immunogens or vaccines. In most cases, the precise mechanisms underlying the effects are unknown. Recently, there is a growing understanding that all known adjuvants function by affecting core adjuvant-responsive genes, but they may differ significantly in their signature responses [18, 140]. These studies suggest that a better strategy to augment vaccine efficacy would be to incorporate a cocktail of adjuvants in the vaccine formulations, rather than a single adjuvant chosen empirically. The mixture of adjuvants containing two or more compounds would complement or modulate individual effects with a broader and hopefully more beneficial impact on the host microenvironment and consequently on vaccine efficacy.

The making of adjuvant cocktails is not easy to achieve. One approach is to consider the modes of action of constituent adjuvants, but that is not clearly understood. An alternative approach would be to use naturally occurring acellular structures such as extracellular matrices (ECMs). ECMs are known to play diverse roles in cellular microenvironments. *In vivo*, they promote cell-to-cell interaction, angiogenesis, and immune extravasations [218-220]. As a biomaterial, they have found wide usage in wound healing and in repair of urinary bladder defects, cardiovascular tissues, and ligament damages, etc.[221-225]. One such acellular ECM is SIS, a biomaterial from porcine small intestinal submucosa (cook Biotech, USA). It consists of predominantly collagens plus glycosaminoglycans, proteoglycans, fibronectin, b-FGF, and TGF-β, to name a few components [226-228]. Even though SIS is of xenogenic origin and thus considered an allograft, it has over several years evoked little, if any [229]. Its unique properties lie in its composition mentioned above; these constituents are highly conserved proteins and are either bioresponse modifiers or promote such responses. As a consequence, wound healing proceeds with tissue granulation and epithelization without the attendance of graft-versus-host reactivity [230, 231]. Most importantly, the particulate nature of SIS makes it readily amenable to phagocytosis by dendritic cells (DCs), which are the most efficient antigen-presenting cells (APCs), and hence, SIS is an attractive candidate as a cocktail of naturally occurring adjuvants.

Studies with SIS xenografts have revealed that when implanted SIS elicits a vigorous immune response but the response is restricted to the Th2 pathway, which is associated with acceptance and remodeling of the graft material [232, 233]. Indeed, the Th2 dominance promotes efficient remodeling possibly by attenuating the pro-inflammatory cytokines induced by the Thl pathway. Recently SIS has been shown to enhance anti-prostrate tumor immunity

evoking effective cell-mediated immunity [234]. Thus it is becoming increasingly evident that commercial SIS preparations could have a broader appeal as an adjuvant and in the making of conjugate vaccines in a larger context. To determine whether this xenogenic product is as good an adjuvant as alum or our terpenoid phytol-derived adjuvant PHIS-01, we asked the following questions: 1) does SIS influence host microenvironment in terms of chemokines and cytokine milieu in a similar fashion at the same or similar way as does a prototypical adjuvant alum?; 2) does SIS involve the so-called core-adjuvant genes as does alum and PHIS-01 in recruiting innate immunity via inflammasomes?; and 3) does SIS enhance antibody responses to soluble, non-self-protein vaccines and hapten-protein conjugate (as a prototype of conjugate vaccines)?.

In this study, the evaluation of SIS adjuvanticity was carried out in BALB/c and C57BL/6 mice via intraperitoneal and subcutaneous routes in the presence and absence of ovalbumin and phthalate-KLH immunogens. Inflammatory responses were studied by microarray profiling of cytokines and chemokines and by qPCR of inflammasomes-related genes. Results demonstrated that SIS provokes neither pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) nor NLRP3 inflammasomes, a molecular platform that is required for caspase-dependent cleavage of cytokines such as IL-1β, IL-18 and IL-33[60, 61], but it does up-regulate IL4 and CD30-ligand. SIS also activates chemotactic factors LIX and KC (neutrophil chemotactic factors), MCP 1 (monocyte chemotactic factors), MCSF growth factor and MIP 1-α (macrophage chemotactic factor), Fractalkine , and Lympotactin, but these response is not as vigorous as with alum. Despite these differences in cytokine elicitation, SIS seems to be an intrinsically strong inducer and is as effective as alum and PHIS-01 in engendering a lasting and specific antibody response, primarily of IgG1 type. Moreover in spite of its proteinaceous nature, SIS evokes very little anti-DNA response, a hallmark of autoimmunity. It appears that

both alum and SIS enhance Th2 type immune response irrespective of the nature of antigens, but in contrast to alum, SIS does not provoke NLRP3 inflammasomes.

Materials and Methods

Vaccine Formulation

Vaccine formulation consisted of either ovalbumin (OVA) (100 µg/mice) or phthalate-KLH conjugate (100 µg/mice) prepared as described by *Ghosh et al* [150]. The antigens were emulsified with two commercial SIS preparations (SIS-H and SIS-M) provided by Cook Biotech as follows: 200 µL of antigen (100 µg) + 5 mg (SIS-H or SIS-M) in 250 µL PBS containing an emulsifier 15% Arlacel A. For parallel comparison, commercial adjuvant such as alum and CFA/IFA or no adjuvant were used. Adjuvants were used as described by manufacturer (Sigma Chemical Co., St. Louis, MO).

Immunization

Female BALB/c, C57 BL/6 mice, 6-8 weeks of age, were used throughout this study. Mice (n= at least 6 in each experiment) were grouped as **(1)** PBS group (only antigen but no adjuvant) (2) CFA/IFA (antigen plus adjuvant, first CFA, then IFA in subsequent immunizations), (3) Alum (4) SIS-H; (5) SIS-M. Vaccine preparations were injected into mice either intra-peritoneally (I.P) or subcutaneously (S.C). Mice were given two booster immunizations at 10-day intervals and a third one 4 months later. 5 days after each immunization, mice were bled under anesthesia through retro-orbital plexus. Serum antibodies induced were assayed by ELISA. For cytokine and chemokines analysis or RT-PCR, mice (n=3) were given the above adjuvants I.P in 500 uL PBS alone without Arlacel A, and peritoneal lavages were collected 24 later.

ELISA Analysis

Determination of levels of anti-phthalate, anti-KLH, or anti-OVA antibodies was assessed by enzyme linked immunosorbant assays (ELISA).Flat-bottomed corning plates were coated for 2 hrs at 37 ºC with 50 µL of 10 µg/mL of either phthalate-BSA conjugate, KLH or OVA. The plates were washed four times with PBS containing 0.01% Triton X-100, blocked overnight with 1% BSA and washed again. Various dilutions (10^1-10^5) of test sera (individual mice sera) from normal and immunized mice were added in triplicate to the plates, and incubated at 37°C for 1 hr. Following incubation and after washing four times with PBS/Triton X-100, rabbit anti-mouse immunoglobulin-horse-raddish peroxidase (HRP) (50 µL) (at 1: 3000 dilutions) was added. Plates were incubated for 1hr and washed again. The rabbit anti-mouse immunoglobulin-HRP was detected by addition of o-phenyl diamine (OPD). The reaction was stopped by adding 50 µL of 10% H_2SO_4, and the intensity of color was determined at OD 490 nm.

To determine anti-DNA antibodies, ELISA plates were pre-coated for 2 hrs at 37 ºC with 50 µl of methylated-BSA (50 µg/mL). The plate were washed four time, and œated with calf thymus DNA (10 µg/mL), and incubated for 2hrs at 37C, and ELISA experiment was carried as described above.

To determine isotypes of antibodies produced (IgM, IgG1, IgG2a, IgG2b, and IgG3) individual mice sera from different groups of immunized mice, were diluted to 1/1000, and then tested in triplicate according to the manufacturer's protocol (Southern biotech, Alabama). Plates were coated with either phthalate-BSA, or OVA as described above.

Analysis of Cytokines and Chemokines

BALB/c mice were injected with adjuvants, and peritoneal exudates were collected after injecting with 3 ml of PBS; peritoneal exudate cells (PECs) were spun down and used for gene expression profiling by RT-PCR. Collected Fluid (1ml) was analyzed for cytokine/ chemokine elicited, using mouse inflammatory cytokine array kits and protocols from RayBiotech Inc. Signal intensities were quantified and analyzed from the array image using Image J software provided by NCBI [152]. Positive and negative controls from six array spots were used to normalize the results from different membranes that were being compared. For each spot, the net optical density level was determined by subtraction of the background density from that of the sample spots, divided by the values of positive control density. Levels of cytokine described in here are expressed as relative intensity (RI) percentages to positive control provided in the membranes.

Analysis of Gene Expression at Injection Sites by Quantitative RT-PCR Array

PECs isolated were used for profiling of gene expression by real-time qRT-PCR. Briefly, total RNA was isolated from all samples according to the manufacturer's recommendations (Ambion, Austin, TX). The RNA preparation was considered to be of good quality if the 260/280 and 260/230 ratios were close to 2. Equal amounts of RNA (1 µg) from all sample groups were reverse-transcribed using the RT2 first-strand kit from SA Biosciences, Frederick, MD, USA. The cDNAs were then labeled by RT2 Real-Time SYBR Green PCR Master Mix (Cat# PA-011, SA Biosciences) as indicated in RT2 Profiler PCR Array protocol (PAMM-033,SA Biosciences). A 25 µL aliquot of this mix was loaded onto the wells of PCR Array plates (PAMM-97, SA Biosciences) and PCR was performed on a Stratagene Mx3000P cycler using the cycling program provided by the manufacturer. Relative changes in genes

expressions were calculated and analyzed using SA Biosciences' web-based PCR Array data analysis methodology (SA Biosciences, http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php website). Gene expressions were normalized with respect to all five house-keeping genes include in the array and calculated as averages of log2 ratios. The array evaluated expressions of 84 genes involved in inflammasome pathway. Genes of mice that differed by 1.5-fold compared to buffer control were considered adjuvant-core response genes.

Statistical Analysis

One-way ANOVA and Student's t-test (SPSS software) were used to determine statistical significance. Levels of $p < 0.05$ were considered statistically significant. Data are expressed as mean ± SD.

Results

Chemo-attractants and Cytokines Elicited at Intraperitoneal Sites in Response to SIS Adjuvants With or Without Ovalbumin Immunogen

Wound healing and tissue remodeling are facilitated by SIS biomaterial possibly by virtue of its ability to initiate and sustain a favorable cytokine milieu [1]. To assess whether inflammation plays a role in the action of SIS, we determined the levels of relevant cytokines and chemokines in peritoneal exudates 24 hours after intraperitoneal injection injection of alum, SIS-H and SIS-M with or without an immunogen, ovalbumin. The results in Figure 22 show that both SIS preparations, SIS-H and SIS-M were similar to alum in inducing a plethora of cytokines and chemokines, including leukocyte chemotactic factors (KC, LIX, eotaxin-2, MCP-1, lymphotactin, Fractalkine, MIP-γ), Macrophage chemotactic factor (MIP-1α), eosinophils chemotactic factor (eotaxin-2), Fractalkiline and FAS L. Clearly, this implies recruitment and

activation of neutrophils, eosinophils, monocytes, and macrophages. There were also modest increases in all experimental groups in the levels of growth factors GM-CSF, M-CSF, G-CSF, which are necessary for differentiation of monocytes to DCs, macrophages, and granulocytes respectively [199-201]. Importantly, the differences in these effects due to alum or SIS are statistically insignificant. Induction of moderate levels of IL-1α and sTNF RI from SIS preparations and alum suggests that there might be some tissue injury. However such injury seemed minor due to the facts that there was no attendant increase in pro-inflammatory cytokines such as IL-6, IL-1β, IL-10, TNF-α and IL-17. The influence of SIS preparations on cytokines associated the adaptive immunity indicated induction of both Th1 and Th2 cells. The SIS preparations evoked Th2 cytokine (IL-13, IL-4), as well as Th1 polarizing cytokine (IL-12P70P40 and IL-12 P40). SIS products also induced a slight increase in cytokines necessary for stimulation and growth of T helper cells including IL-2, IL-9 and CD40 L.

Importantly, when adjuvants were assessed in combination with the antigen ovalbumin, there was no much impact on inflammatory environments already induced by SIS or alum adjuvants described above (Figure 23).

Relative Modulation of Inflammatory Gene Expression at the Peritoneal Site by SIS and Alum with or without Ovalbumin Antigen

Innate immunity is known to be strategically involved in initiating inflammatory processes as a response to ‘stranger or danger signals’ from adjuvants and antigens. We hypothesized that SIS like alum might function by interacting with the receptors of the innate immunity system, particularly those implicated in inflammatory processes, such as Nod-like receptors (NLRs). In recent reports, NLR-associated gene activation pathways have been shown to play crucial roles in the adjuvanticity of alum and MF59 [192, 235]. To address

whether adjuvanticity of SIS follows the same pathway, we focused on gene expression profiling of NLRs in mice. Using an RT-PCR microarray, we measured the expressions of 84 genes immunized I.P with SIS or alum alone or in combination with OVA (Table 1S). Our results in Figure 24 indicate that SIS alone like alum significantly activated core inflammatory-response genes. However, SIS differed from alum and affected only 17 genes, whereas alum modulated 37 genes. Among the core-response genes modulated by SIS, there were 11 genes up-regulated above 1.5 fold on a log2 scale (Figure 24A) and 6 genes down-regulated below 1.5 fold (Figure 24B). Genes responding to SIS included Nod-like receptors NLRP4b, NLRP 5 and NLRP9b, and cytokine genes associated with Th-1 response such as INF-γ, IL-12b, INF-β and IL-18. In addition, SIS up-regulated the expressions of inflammatory genes such as caspase 12 and TNFSF 4 and 14. In contrast to SIS, alum additionally promoted the up-regulation of several NLRP genes including NLRP3, as well as Txnip and Pstpip that were shown previously to participate in NLRP-3 inflammasomes [208, 236]. Moreover, alum, unlike SIS, activated a Th2-related cytokine gene IL-33, chemotactic factors CCL12 , CCL7 and CXCL3, a downstream signaling factor for MAPK pathway , and interferon-regulatory factor IRF; the latter two both could be involved in the production and regulation of pro-inflammatory cytokines. Furthermore, besides up-regulating Nod-like receptors SIS and alum both increased IL-1β expression. When combined with OVA as the antigen, neither alum nor SIS did greatly modify the expression of the aforementioned gene profiles (Figure 25). These adjuvants by themselves also up-regulated Ptgs2, a genes associated with inflammation (Figure 25A), addition of OVA in the formulation did not have any significant impact on this gene. However, when ovalbumin was mixed with alum or SIS, both NLRP4e and NLRP6 genes were

upregulated. Besides up-regulation both adjuvants also down-regulated a number of genes (as shown in figure 25B), and combination with OVA had no further down-regulation of genes.

SIS Adjuvants Promote Higher Antigen-Specific Antibody Response than Alum and Promote T Helper Type-2 Like Responses in C57bl/6 Mice

The foregoing study indicates that both SIS and alum would influence host microenvironment in terms of chemokines and cytokines, although there are characteristic differences. This led us to address whether this ability to act on innate immunity-related genes would help augment acquired immunity and thus vaccine efficacy. Using OVA as the model antigen in our vaccine formulations, SIS and alum were compared for their effectiveness in induction of anti-OVA antibody in C57Bl/6 mice. The results in Figure 26 revealed significant booster effects; clearly SIS and alum were both highly effective in increasing the magnitude and the titer of OVA-specific antibody, particularly high levels of IgG1 antibody subclasses. This suggests that both SIS and alum promoted a Th-2 response. This result is in agreement with previous reports implying that SIS would bolster Th2 environment and mild inflammatory response when used as the biomaterial for wound healing purposes [232]. In comparison, alum seemed to be more inflammatory.

SIS Adjuvants Promote Antigen-Specific Antibody Response but no Measurable Autoimmune Effects

Next, we addressed whether the adjuvanticity of SIS biomaterials would vary depending on mouse strains, routes of immunization and antigenic differences. In addition, we tested whether immunization with SIS would cause or aggravate autoimmune responses, a side effect associated the use of many adjuvants [135]. This study was performed in non-immune prone BALB/C mice using phthalate-KLH conjugate as experimental antigen by both intraperitoneal

and subcutaneous routes. Previously we showed that phthalate, a plasticizer and a common environmental hazard, causes lupus-like syndromes in NZB/WF1 mice, and that response is greatly influenced by inclusion of certain adjuvants [141, 171]. The response to phthalate-KLH is characterized by an antibody response that is mostly directed toward the phthalate moiety in the conjugate, as well by induction of cross reactive anti-DNA antibodies. In BALB/c mice there also was an increase in anti-DNA antibody, but it was down-regulated after subsequent booster immunizations. Our results as shown in Figure 27 indicate that, irrespective of routes of immunization, BALB/c mice immunized with phthalate-KLH in emulsion with SIS plus arlacel (an emulsifier) exhibited a high titer antibody response. The magnitude of this response was not different from that evoked with alum, and phthalate cross reactive anti-DNA antibody levels were insignificant in mice immunized with both SIS and alum.

Analysis of IgG isotype directed against phthalate moiety also showed that SIS adjuvants like alum and CFA/IFA promoted IgG1 type class by both I.P and S.C routes. However, compared to SIS, alum was more efficient in inducing a robust IgG2a and IgG2b responses particularly after I.P. injection (Figure 28).

Discussion

This study evaluated the adjuvanticity of an acellular biomaterial derived from porcine small intestinal submucosa (SIS), a commercial product licensed primarily for use in surgical procedures dealing with wound healing in humans [221, 223, 224]. Despite being xenogeneic, SIS works well *in vivo* without causing concerns for detrimental inflammatory effects. Indeed, it greatly facilitates the process of wound healing. Though largely a collagenous product, SIS is known to have minute amounts of other protein constituents associated with such extracellular matrices [226-228]. Nevertheless, this highly sterile biomaterial has been of interest for use as

a type-of multi-protein, naturally occurring, cocktail adjuvant since it proved effective in the successful development of a model prostate cancer vaccine [234].

Immune enhancement by adjuvants, however, can work both ways: it could be ameliorating or detrimental. Therefore for a biomaterial or a compound to be adjuvant, safety is certainly the primary concern. One way to assess safety is to determine whether the test material causes physical or behavioral problems such as splenomegaly or discomfort. In the immunological context, the safety issue can be better evaluated in terms of important parameters associated with inflammatory response. This latter assessment serves two purposes. It helps evaluate inflammatory or immunodulatory cytokine/chemokine microenvironment as well as assess the activation in sequence of innate and acquired immunity. We tested the safety and efficacy of two batches of SIS biomaterials from Cook biotech, SIS-H and SIS-M (named this way just to distinguish them as being from different lots) alone and in combination with soluble protein antigens in inbred strains of mice, C57BL/6 and BALB/c. First we evaluated the impact of SIS biomaterial on host micro-environment (cytokines and chemokines), a property commonly associated with a known adjuvant like alum. Having evaluated SIS on this parameter, we determined its effect on humoral immunity by assessing the quality and magnitude of specific antibody response.

Our study of chemokine/cytokine milieu reveals that SIS does host microenvironment, albeit to a lesser degree than alum. The latter activates more genes including NLRP3 inflammasome, a molecular platform that is required for caspase-dependent cleavage of cytokines such as IL-1β, Il-18 and IL-33[60, 61], which activates strong inflammatory response. In contrast, SIS up-regulates IL-1β but it does not affect NLRP3. SIS activates some other NLRP genes such as NLRP4b, NLRP5 and NLRP9 and up-regulates their expressions.

However, physiological roles of these Nod–like receptors in inflammation are not understood. Interestingly, SIS does not evoke pro-inflammatory molecules such as IL-6, TNF-α and IL-17. However, both SIS and alum up-regulate some common core-adjuvant response genes, such as LIX and KC (neutrophil chemotactic factors), MCP 1 (monocyte chemotactic factors), BLC (B lymphocyte chemotactic factor), and MIP 1-α (macrophage chemotactic factor). In addition, SIS adjuvants as much as alum promoted expression of growth factor; GM-CSF, G-CSF, M-CSF necessary for the differentiation and maturation of monocytes and granulocytes that develop to mature antigen presenting cells [199-201]. Alum reportedly has the ability to activate monocytes derived APCs that in turn would isotype switching and higher-titer antibody response [39, 237], alum also recruits granulocytes may to play an auxiliary role in enhancing this response [116]. From our results, it appears that SIS adjuvant is much like alum in provoking monocytes and granulocytes, and this may explain how it enhance antibody response. Furthermore, recruitment of APCs following SIS administration is accompanied by production of cytokines such as IL-4 and CD 40L, which are crucial for priming of CD4+ T cells and consequently for isotype switching that occurs during B cell response [238, 239].

We previously showed that phthalate conjugates can induce cross-reactive anti-DNA antibody; this response is magnified by some adjuvants like leading to lupus-like syndromes in rodent [142, 171]. Thus, SIS activates a chemokine/ cytokine milieu that accompanies engagement of innate immunity. It also enhances antibody response of different Ig classes implying activation of specific T helper cells. In this study, SIS biomaterials not only significantly enhanced anti-OVA and anti- phthalate responses, but also promoted IgG1 subclass, like alum indicating induction of Th2 response. This Th2 response is seen when SIS or alum is used in combination with antigens. However, when tested alone, SIS adjuvant was

able to up-regulate the gene expression of Th-1 polarizing cytokines such IL-18[240], implying that the xenogeneic SIS could facilitate a Th-type1 response in intracellular environment. Effectively, a previous report by Suckow et al also shows that when xenogeneic prostate tissue vaccine, it stimulates a Th1 response and prevents prostate cancer growth [234, 241].

Together, these properties would make SIS material a useful adjuvant in formulations of soluble protein vaccines as well as conjugate vaccines like the one used against childhood pneumococcal infections. The ability of SIS to activate innate immunity appears to be closely related to the unique composition of SIS material derived from extra cellular matrix (ECM).

In conclusion, SIS adjuvant augments immunogenic potentials of soluble proteins without inducing any pathogenic inflammatory response. It is safe and effective. Most commercial adjuvants are known to function by inflicting tissue damage at sites of injection through inflammation. This may have unintended consequences such as autoimmune disorders [121]. This is a drawback of established adjuvants like complete Freund's adjuvant, which causes inflammation, tissue necrosis, with formation of granuloma in lung and kidney [121]. Squalene in MF59 and alum also induce inflammatory responses; while squalene is linked to autoimmune responses in rodents [135] and alum to dementia [38]. In this respect, our studies establish that SIS which has a cleaner record as a biomaterial for several years is truly an effective adjuvant and unlike alum it causes little, if any, inflammatory response.

Figure 22.

Chemokines and Cytokines Profile Induced by ECM SIS Alone.

BALB/c mice were injected i.p. with SIS-H or SIS-M, and peritoneal exudates harvested after 24 hr (B). Control groups were treated with either the standard adjuvant alum or PBS buffer. Peritoneal fluids were assayed to determine chemokines and cytokine expression as detailed in Materials and Methods. Data are expressed as the mean relative intensity relative to the positive control of each chemokine or cytokine protein, detected using pooled peritoneal fluids of 3 mice per group in duplicate. The result is the average of two separate experiments.

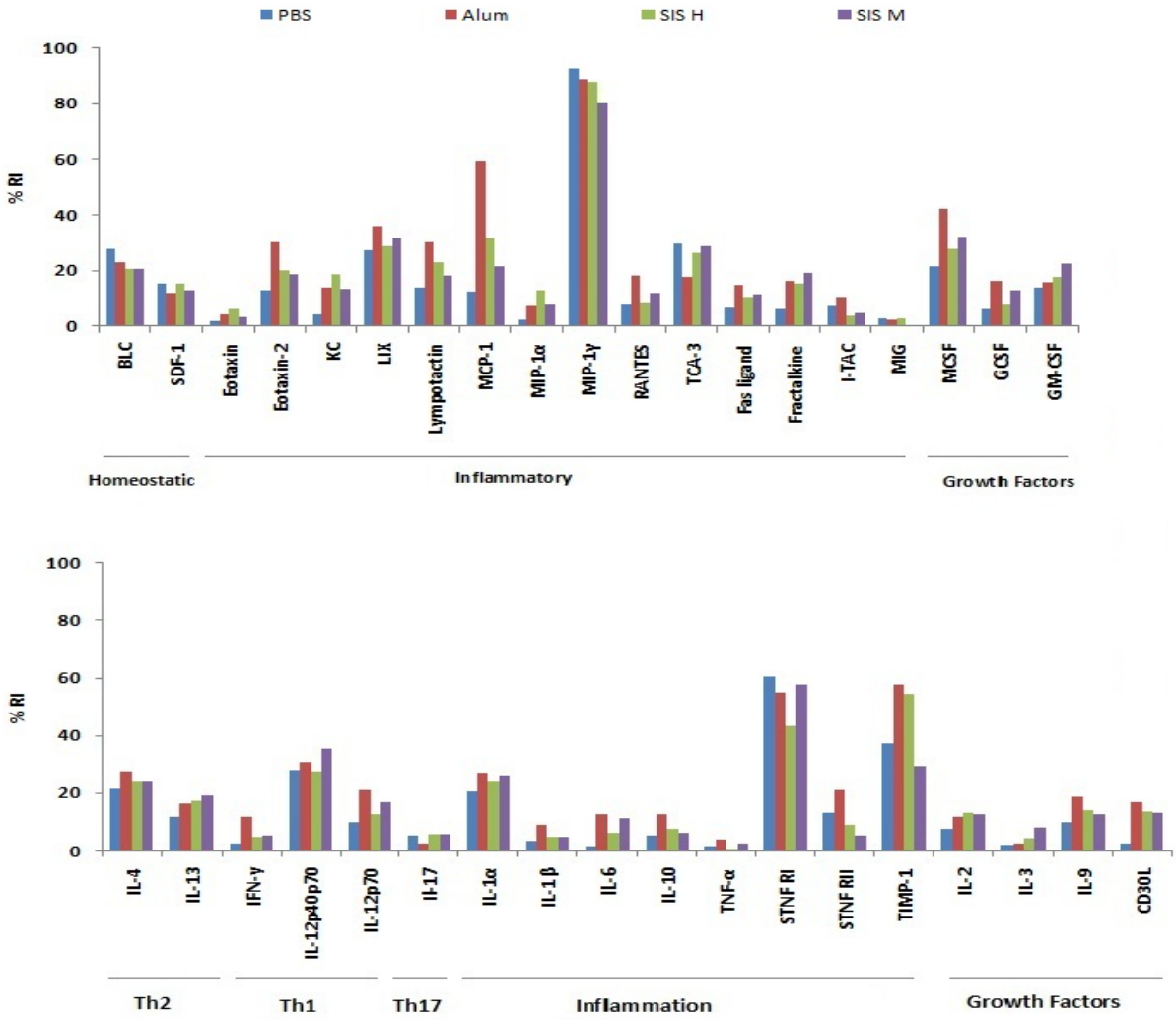


Figure 23.

Chemokines and Cytokines Profile Induced by ECM SIS in Inoculums with OVA. BALB/c mice were injected i.p. with SIS-H or SIS-M in inoculums with OVA and peritoneal exudates harvested after 24 hr (B). Control groups were treated with either the standard adjuvant alum in inoculum with OVA or OVA alone. Peritoneal fluids were assayed to determine chemokines and cytokines expression as detailed in Materials and Methods. Data are expressed as the mean relative intensity relative to positive control of each chemokine and cytokine protein detected using pooled peritoneal fluids of 3 mice per group in duplicate.The result is average of two separate experiments

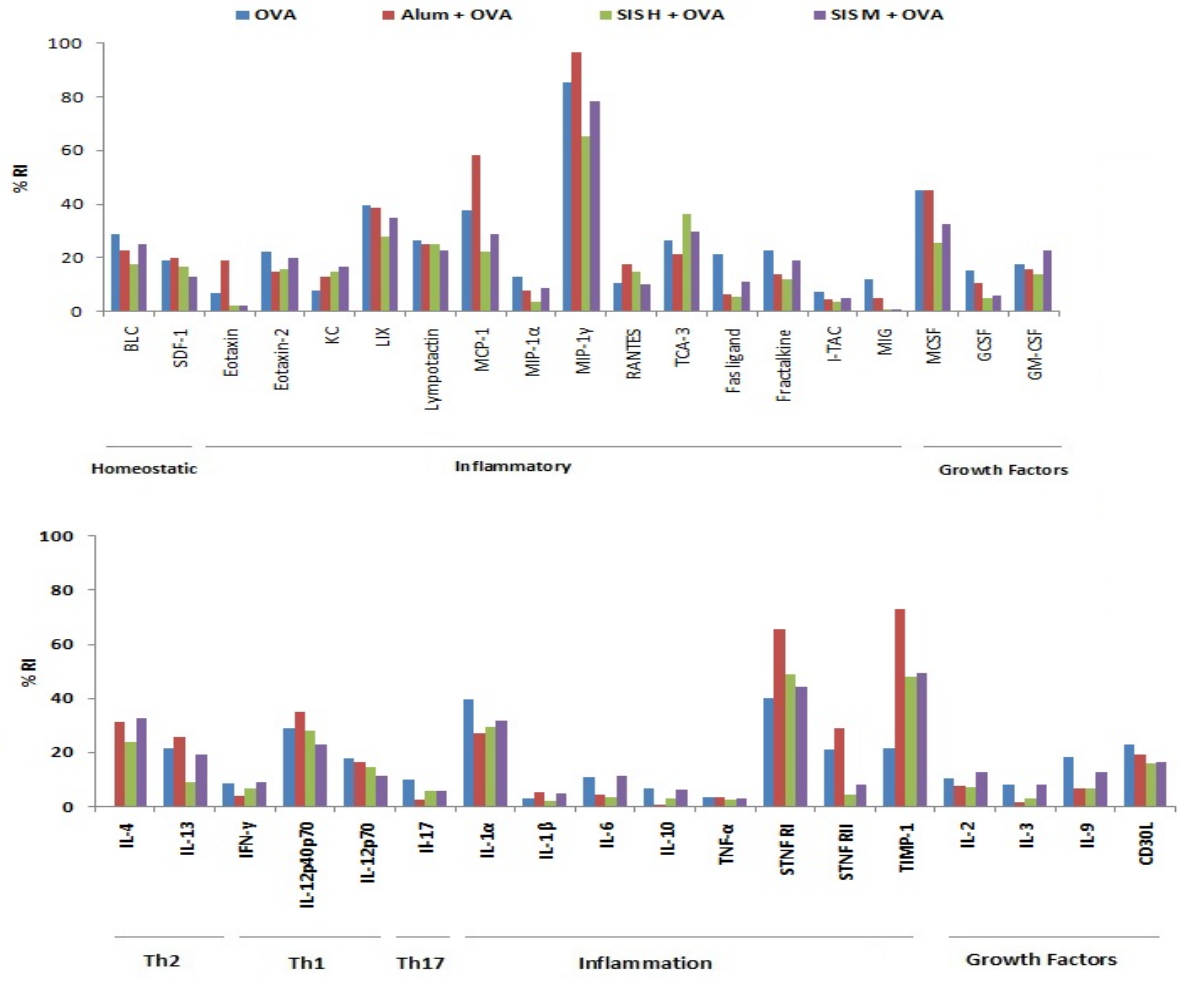
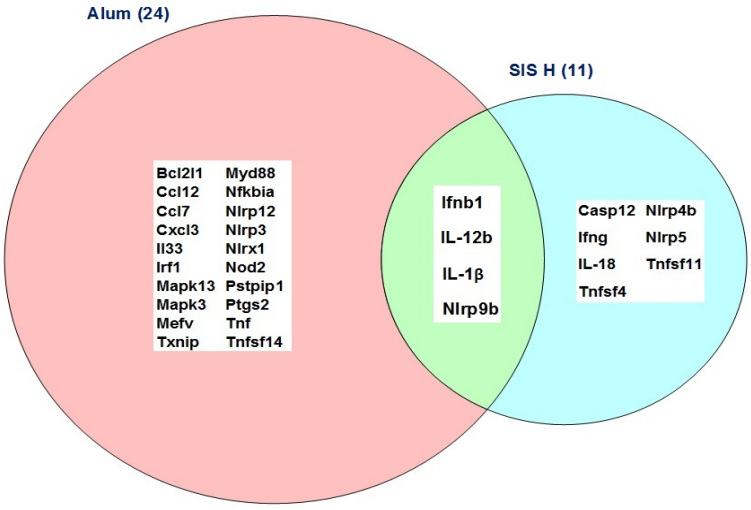


Figure 24.

RT-PCR Microarray Analysis of Transcriptome Profiles of Inflammatory Genes Induced by Vaccine Adjuvants SIS-H or Alum, in Mouse Peritoneum.

Adjuvants were not combined with other material; Genes (84) were assessed and those genes up-regulated (A), or down-regulated (B) with an average $\log_2$ ratio $\geq \pm 1.5$ were selected and plotted as a Venn diagram.

A



B

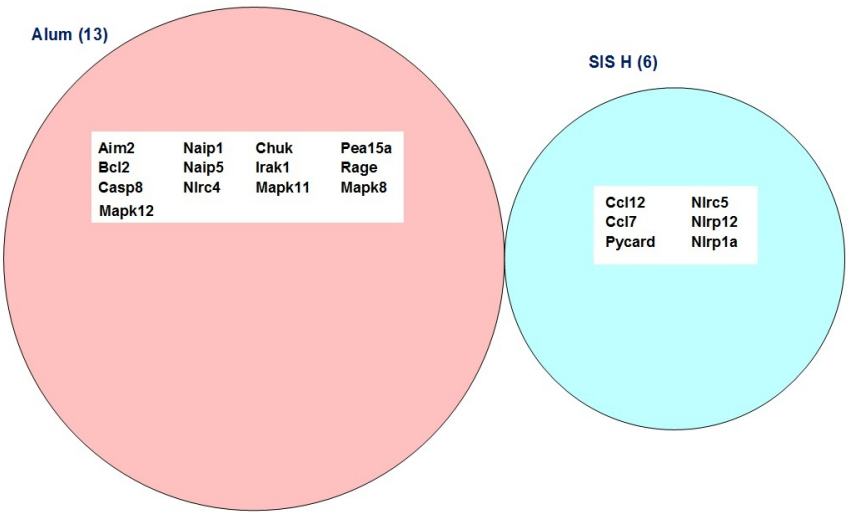
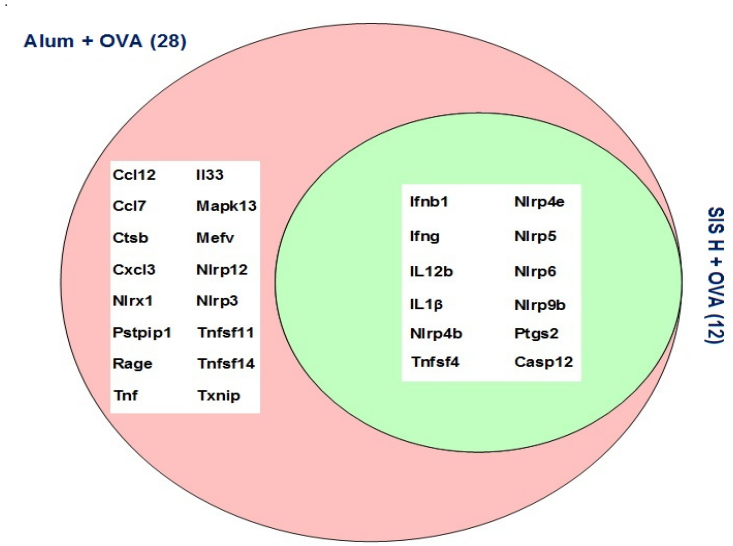


Figure 25.

RT-PCR Microarray analysis of transcriptome profiles of inflammatory genes induced by vaccine adjuvants SIS-H or alum in combination with ova in mouse peritoneum. Genes (84) have been tested and only genes up-regulated (A), or down-regulated (B) with an average log2 ratio ≥ ±1.5 were selected and plotted as Venn diagram.

A



B

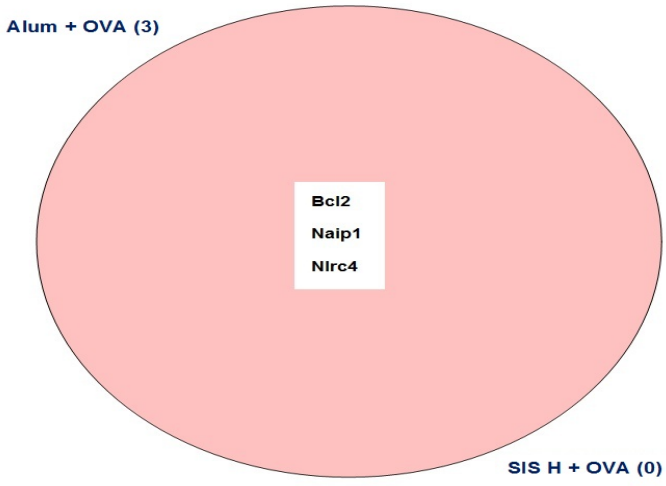


Figure 26.

Effects of SIS Adjuvants on Anti-OVA Antibody Response in C57 Black/6 Mice.

Mouse serum samples were collected on day 5 after (A) 2nd immunizations or (B) 3rd immunization with OVA plus adjuvants as described under Materials and Methods. Antibody responses were assessed using ELISA. (C) Determination of IgG sub-classes of anti-OVA antibodies induced with OVA in different adjuvants after 3rd immunization. This was done in serum samples (Dilution 1:1000) using commercial ELISA isotyping kits. The results represent mean ± SD (n = 6 mice per group in two separate experiments). The significance in experimental groups was determined relative to the group given antigen only (no adjuvant group) at the level of $p \leq 0.05$.

A

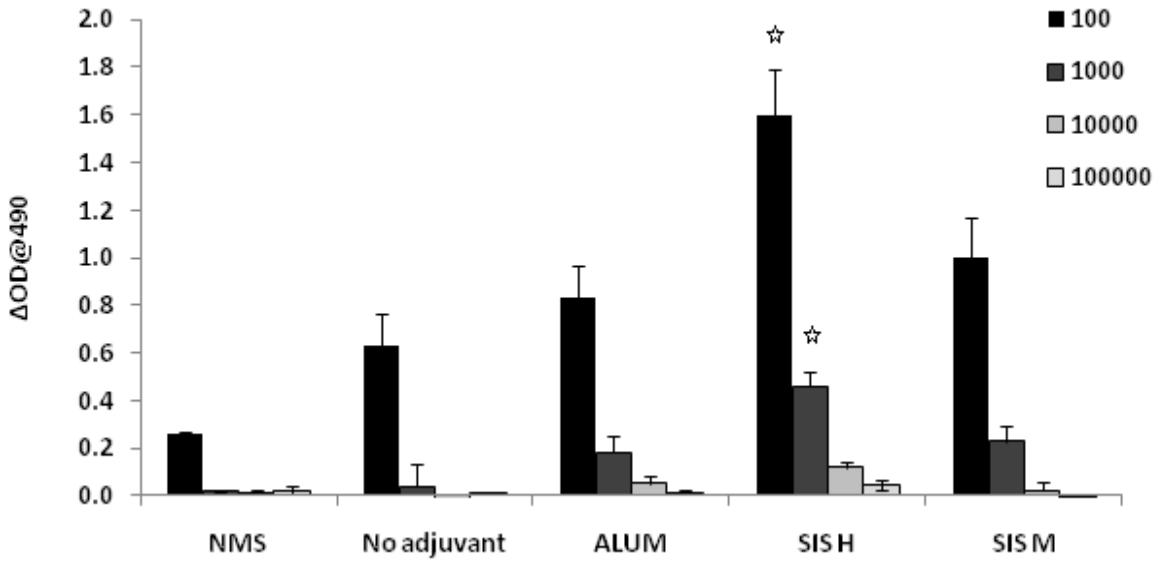
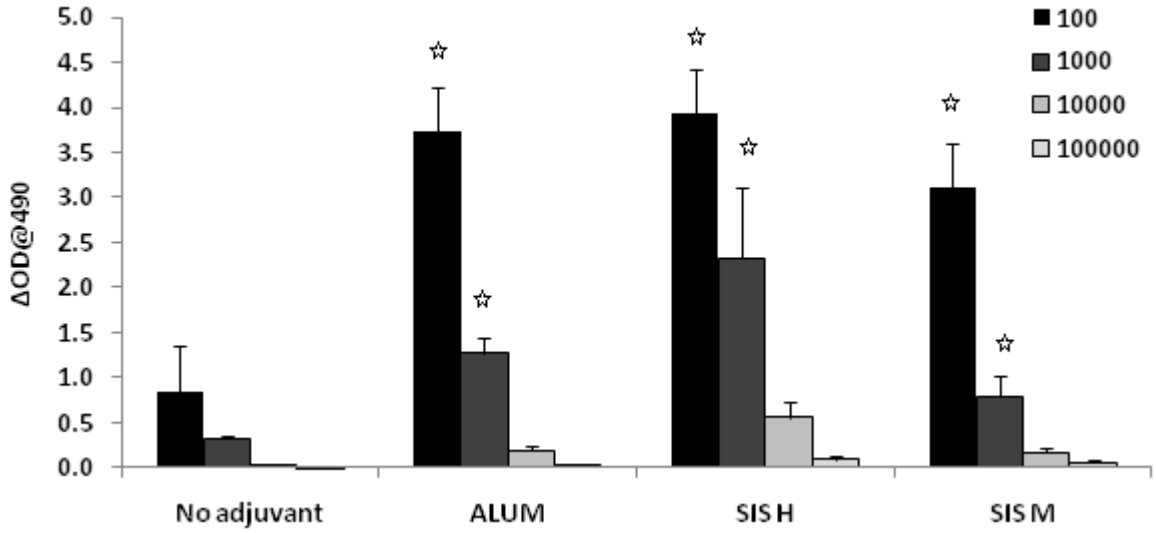


Figure 26 (Continued).

B



C

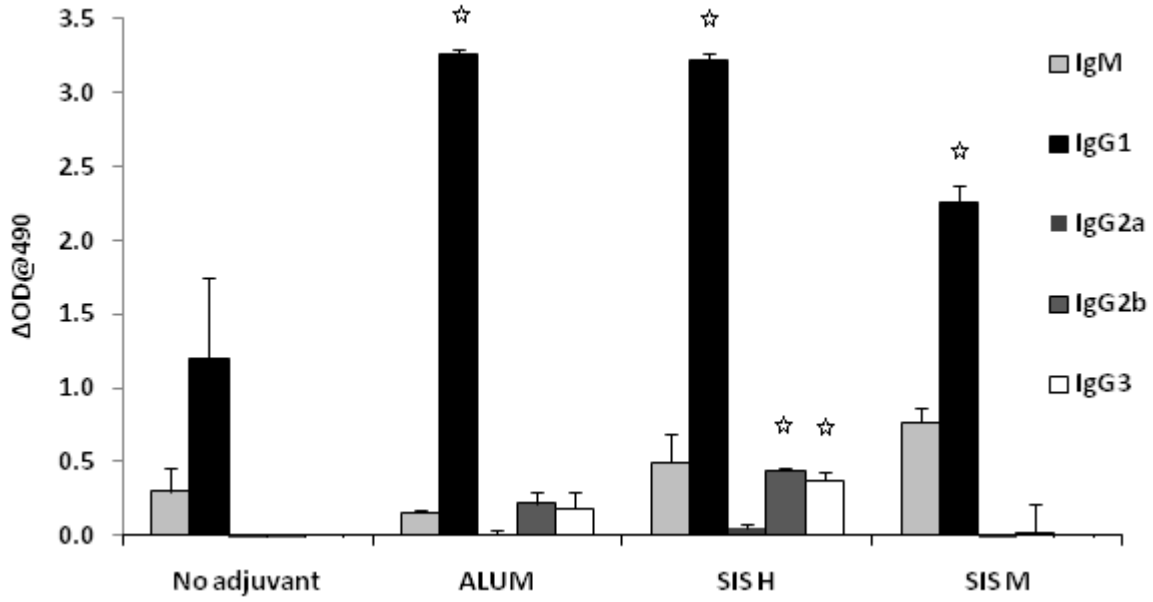


Figure 27.

Evaluation of Phthalate-KLH Antibody Response in BALB/c Mice.

Mice were immunized with phthalate-KLH conjugate emulsified in different adjuvants. Serum samples were collected as described under Materials and Methods and diluted to 1:1000 in 0.5 % PBS/BSA. Anti-phthalate, anti-KLH, and anti-DNA antibody levels were determined using ELISA. The results represent mean ± SD (n = 6 mice per group in two separate experiments). The significance in experimental groups was determined relative to the group given antigen only (no adjuvant group) at the level of $p \leq 0.05$.

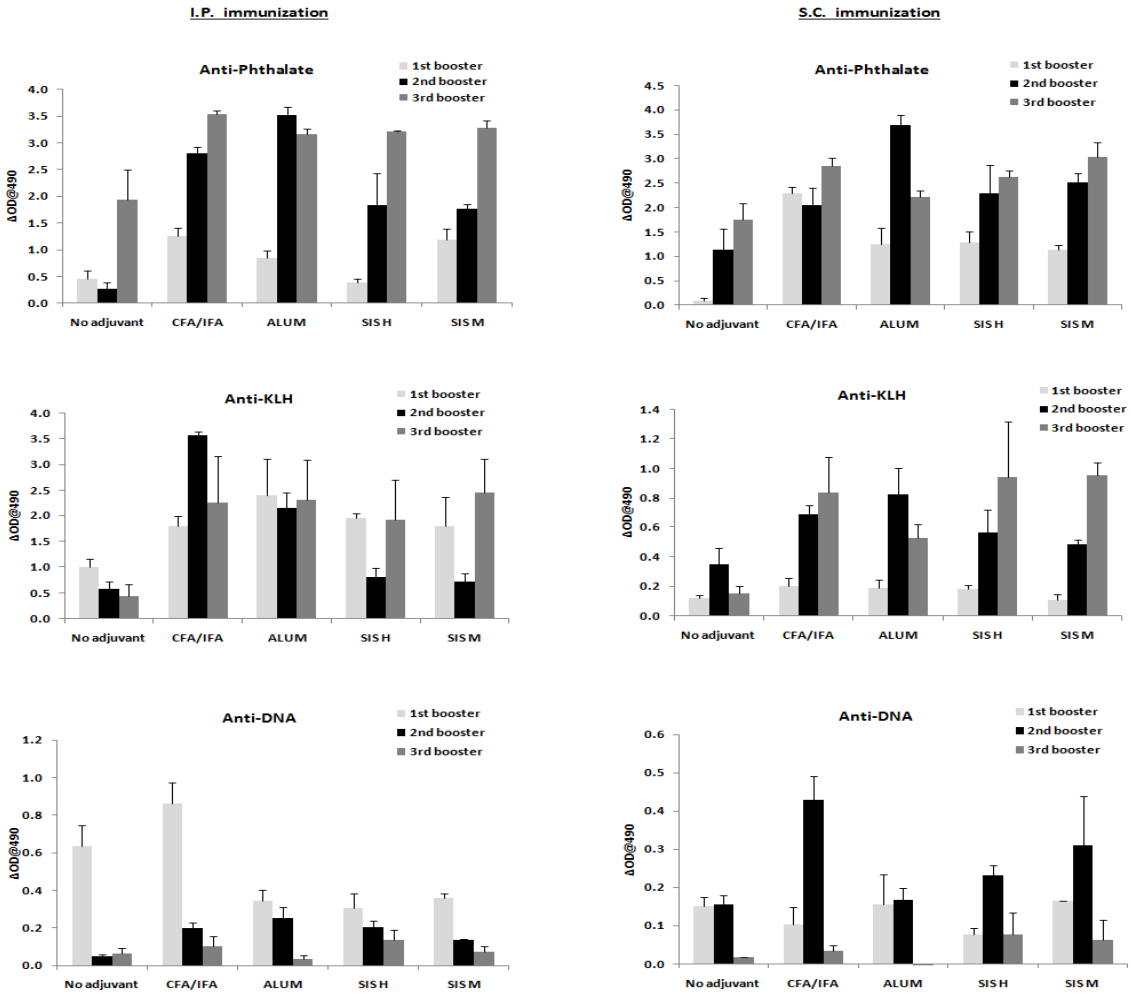
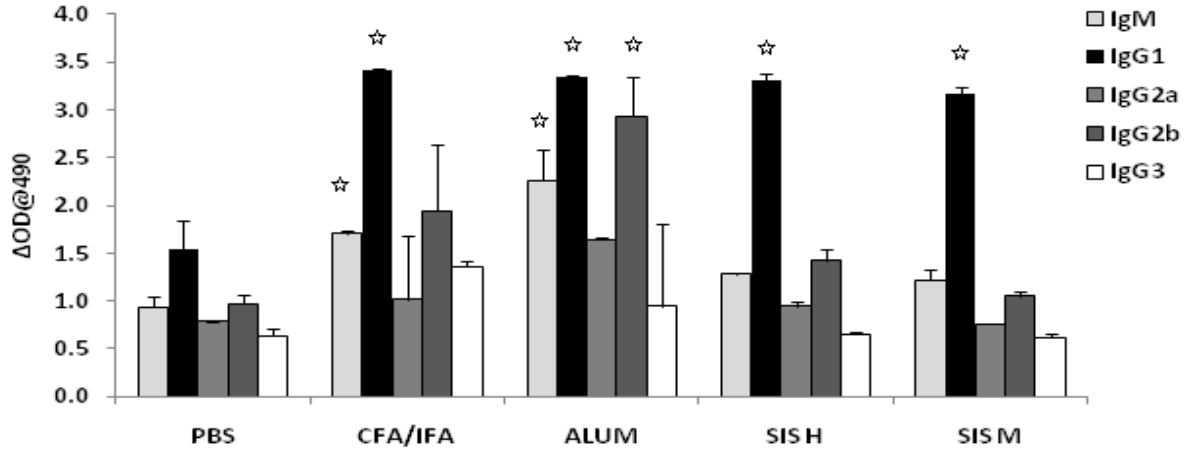


Figure 28.

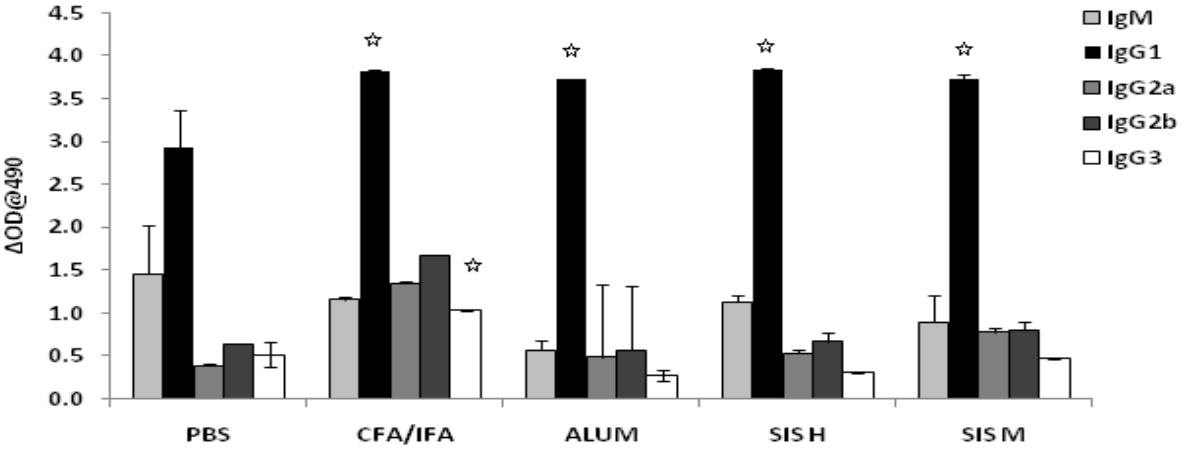
Determination of IgG Sub-classes of Anti-phthalate Antibodies Induced with Phthalate-KLH Conjugates in Different Adjuvants.

This was done in serum samples (dilution 1:1000) collected after (A) interperitoneal immunization or (B) subcutaneous immunization, using commercial ELISA isotyping kits. Results represent mean ± SD (n = 6 mice per group in two separate experiments). The significance in experimental groups was determined relative to the group given antigen only (no adjuvant group) at the level of $p \leq 0.05$.

A



B



CHAPTER 6

TOPIC 4: SYNTHETIC ADJUVANTS EFFECTIVENESS IN AUTOIMMUNE-PRONE NZB/W F1 MICE

Abstract

The objective of this study is to the assess safety and efficacy of two terpenoid immunostimulants PHIS-01 (phytanol) and PHIS-03(phytanyl mannose) developed by chemical modifications of phytol. Based on our earlier study in BALB/c and C57BL/6 mice, we contend that these compounds would also be effective as SIS (porcine small intestinal submucosa), a newly introduced commercial adjuvant from Cook Biotech and alum, the standard adjuvant in autoimmune-prone NZB/WF1 mice. The issue is whether the adjuvanticity correlates with the chemical nature of adjuvants or not. In this case, phytol derivatives are hydrophobic, while alum is hydrophilic, and SIS, on the other hand, is essentially a collagenous protein cocktail derived from extracellular matrices. Therefore, it is of interest to determine whether these diverse compounds have common denominators to function as effective adjuvants even in autoimmune prone NZB/WF1 mice without enhancing lupus-like syndromes and detrimental cytokine/chemokine microenvironment. We studied hapten-specific antibody response, anti-DNA response, and other parameters of autoimmune disorder. We also assessed antibody isotype and cytokine/chemokine profile induced. Our

results indicate that phytol-based immunostimulants PHIS-01, PHIS-03 and SIS adjuvants have similar effects as alum in augmenting hapten phthalate-specific antibody without the aggravation of lupus-like syndromes promoted with phthalate. These adjuvants are also effective in down-regulating cross reactive anti-ds DNA Abs triggered by phthalate immunization. Most importantly, Phytol-based adjuvants, SIS and alum all modulate systemic pro/anti-inflammatory cytokines and Th1/ Th 2 balance creating host microenvironment which reduces the onset of autoimmune syndromes in NZB/WF1 mice.

Introduction

Prophylactic vaccination is considered the most cost-effective way to control diseases; however, in recent years, there has been growing doubts about the benefits of vaccines, because, primarily of largely unsupported claims that constituents in vaccine formulations may have long-lasting deleterious effects. These concerns have led to a surge of efforts to redesign vaccines by employment of modern technologies involving recombinant protein antigens, purified allergens, and pathogen-associated offending agents[159]. Alongside, there are new efforts directed to molecularly defined adjuvants or immunostimulants that nonspecifically boosts immunogenic potentials of a vaccine. Once considered “immunologists’ dirty tricks’, adjuvants are garnering considerable attention with regard to their modes of action, safety, and effectiveness. The objective is to overcome the constraints of empiricism in the choice of adjuvants.

Safe and broadly effective immunostimulants are also the goal of this study. This led us to chemically modify the phytol component of chlorophyll to develop different phytol derivatives [141, 142]. Although it can be toxic at high doses, phytol is known for many beneficial effects on animal models [165, 166]. In earlier studies, we observed that modified

phytol compounds such as PHIS-01 (phytanol) and PHIS-03 (phytanyl mannose) are safe and highly effective adjuvants in immunocompetent inbred strains of mice, BALB/c and C57BL/6. They enhance immunogenicity of many soluble protein antigens and also of heat-killed pathogens [141, 142]. In some instances, phytol compounds work better than alum, the widely used adjuvant licensed for human usage [141, 142]. Arguably, not all vaccine recipients are equally immunocompetent. This necessitates evaluation of putative adjuvants alone and in combination with vaccine materials in both normal and compromised subjects.

This study focused on autoimmune-susceptible NZB/W F1mice strains that develop renal pathology, circulating immune complexes and auto-antibodies like anti-ds-DNA antibodies. These immune complexes get deposited in the glomerulus and incite strong immunological and inflammatory responses characterized by production of pro-inflammatory cytokines and chemokines, recruitment and activation of circulating leukocytes, and tissue damage. Despite being immune enhancers, adjuvants could also cause aggravation of autoimmune disorders. An isoprenoid adjuvant pristane has been shown to promote lupus- like syndromes and pathologic nephritis in both autoimmune-prone and non-susceptible mouse strains after a single intra-peritoneal administration [186, 187, 242]. This is in contrast to the effects of isoprenoids phytol and its derivative PHIS-01. Furthermore, squalene, a triterpene and Freunds' adjuvants (CFA/IFA) could also provoke lupus-like syndromes in non autoimmune- prone BALB/c mice[11]. Obviously these adjuvants in a vaccine would likely be harmful in genetically predisposed or environmentally compromised individuals. In this context, it appears that not only phytol is safer, but most certainly its derivative like PHIS-01 as well [142]. Whether this is true for PHIS-03 (phytanyl mannose), which by virtue of its composition is less hydrophobic than PHIS-01, is not known.

Another adjuvant, SIS (porcine small intestinal submucosa) is collagenous extracellular matrix (ECM) of Cook biotech that is widely used as a non-toxic scaffolding biomaterial in wound healing [221-225]. like some other studies, including ours study, SIS proved to be a highly effective immunoadjuvant in immunocompetent mice strains [234]. Since SIS contains evolutionarily conserved proteins such as collagen and traces of other proteins of ECM, it can be regarded as a cocktail of adjuvants. However, how effective it is in autoimmune prone mice NZB/WF1 has not been previously tackled.

In a previous report, we established that Phthalate, which is plasticizers often used in medical devices and a solvent in cosmetics products, can induce cross reactive anti-dsDNA antibody response in both non autoimmune prone mice (BALB/c) as well susceptible NZB/ W F1 mice. However, only NZB/W F1 mice develop lupus-like syndromes characterized by high levels of antibodies, renal pathology and high mortality rates[171]. In this study, we also used a conjugate (phthalate (phthalate-KLH) as the immunogen in NZB/WF1 mice with or without alum or the experimental adjuvants SIS and PHIS-01 and PHIS-03. This study was undertaken to examine immune-modulatory changes inflicted by choice of adjuvant, which may either down-regulate or aggravate the autoimmune syndromes in NZB/WF1 initiated by phthalate. We specifically addressed whether all these adjuvants (1) induce phthalate-cross reactive anti-DNA response; (2) exacerbate these adverse effects with booster immunizations; and (3) affect host immune microenvironment in terms of systemic chemokines and cytokines.

Material and Methods

Animals and Antigen

Female NZB/WF1 mice 6-8 weeks of age were purchased from Jackson laboratory and were housed in the animal facility of Indiana State University followed under a specific

protocol approved by the Institutional Animal Care and Use Committee (IACUC) of Indiana State University. Ortho-phthalate-protein conjugates were prepared by azo-coupling of diazotized 4-aminophthalic acid (disodium) to KLH as described by Ghosh et al [150].

Immunization Regimen

The phytol derivatives, PHIS-01 and PHIS-03 (US patent pending), were obtained by chemical modification of phytol according to the literature [146-149]. Two batches of newly introduced SIS adjuvants, SIS-H and SIS-M, were provided by cook Bio-Tech, IN. Alum was purchased from Sigma Chemical. The inocula consisted of 200 µL of phthalate-KLH (100 µg/mice) and equal volumes of either PHIS-01 (43mg), PHIS-03(5mg), SIS-H (5 mg in 15% aralcel A, an emulsifier), SIS-M (5 mg in 15% aralcel) or Alum. These ingredients were vigorously mixed a few times in a syringe and by vortexing. Thus, the Inocula prepared were administered intra-peritoneally in a volume of 400µL to six to eight-week old mice (Five mice per group). Mice were given two booster injections at 10 day-intervals and bled 5 days after each immunization through retro-orbital veins. The parallel control groups of mice were immunized with only ortho-phthalate-KLH but no adjuvant. To determine how long the effects of immunizations would persist, the adjuvanted and control groups were administered with just phthalate-KLH, and that followed 5 months after last immunization. Five days after this immunization, mice were bled, sera collected and assayed for antibody response.

Assessment of Serum Levels and Isotype of Anti-Phthalate and Anti-DNA Antibodies

Serum anti-phthalate and anti-DNA antibody responses were determined in triplicates using enzyme linked immunosorbant assays (ELISA), as described previously [171]. Isotyping was done in triplicates using mice sera at 1/1000 dilution using ELISA plates coated with either

phthalate-BSA or calf thymus DNA. Commercial isotype-specific rabbit antisera at 1:500 dilutions were used, and the assay was carried according to the manufacturer's protocol (Southern Biotech, Birmingham AL).

Assessment of Cytokine and Chemokines

Cytokine and chemokine profiles of control and experimental mice were assessed using mouse RayBiotech inflammatory cytokine array kits. Sera collected were diluted 1:5 in the reagent provided with the kits. Detection of cytokine was done according to the manufacture protocols. Membranes were exposed to X-ray films (Kodak X-OMAT AR film), and signal intensities were quantified and analyzed using Image J software from NCBI [152]. Biotin-positive and negative controls at six spots were used to normalize the results from different membranes. For each spot, the net optical density level was determined by subtraction of background density from the sample density and divided by the positive control density. The results were expressed as percentage of relative intensity (RI) of experimental to positive control.

Renal Pathologic Evaluation

At 8month old, Mice were sacrificed, and urine and blood samples were collected. Blood urea nitrogen (BUN) and proteinuria were tested using azostix, and Multistix [171]. Proteinuria and BUN were estimated following the manufacturers' protocols. Kidneys tissues isolated were fixed in 4% paraformaldehyde. Slides were stained using hematoxylin and eosin (H&E). Histology was performed at the laboratory of Dr. Roland M. Khor M.D Chief of Pathology and Certified Pathologist at the Terre Haute Regional Hospital.

Results

Evaluation of Adjuvants in Augmenting Antibody Responses to Phthalate in Autoimmune-Prone Mice NZB/WF1

Anti-phthalate antibody response induced in NZB/WF1 mice (12 week-old) by repeated vaccination was monitored to assess the effectiveness of phytol-based adjuvants (PHIS-01, PHIS-03) compared to alum and SIS. In group of five, Mice received two booster immunizations and then again after 5 months (by then NZB/WF1 mice 32-week old) of resting, they were injected with only phthalate-KLH but no adjuvant. Parallel control groups were exposed to phthalate-KLH with no adjuvant. Results shown in Figure 29 (A, B) reveal that all mice immunized with adjuvanted phthalate-KLH developed significant levels of high-titer antibodies. Moreover, only the adjuvanted groups but not the control non-adjuvanted groups responded with high-levels of serum anti-phthalate antibody to a repeat antigenic stimulation given after a period of five months. Among the adjuvanted groups, PHIS-01-treated group was the best responder in terms of specific antibody response, followed by alum. Responses in PHIS-03 and SIS groups were relatively less robust.

Immunization with phthalate-KLH has previously been shown to evoke cross-reactive antibody to self- ds-DNA [171]. We determined whether adjuvants could influence induction of this cross-reactivity that was previously reported to occur when mice were injected with phthalate-KLH and DEHP [142]. The results in Figure 29 (1D and 2B) show that mice immunized with phthalate-KLH adsorbed to alum developed significantly higher levels of anti-DNA response compared to Phytol or ECM SIS adjuvants after two booster immunization ($p>0.05$). When all groups were rechallenged with phthalate-KLH after 5 month, they experience an upswing in anti-DNA response that varied considerably among adjuvant-treated

groups. The order of response in terms of titer was higher in alum and SIS-H groups, followed by PHIS-01 treated group. Mice immunized with phthalate-KLH emulsified in either SIS-M or PHIS-03 developed less anti-DNA response.

Effect of Adjuvants on Antibody Isotype Profile

The quality of antibody response to both phthalate and ds-DNA in adjuvant-treated groups was further assessed in terms of isotype switching following repeated immunization. The results in Figure 30 (A, B) compare the effectiveness of each adjuvant to modulate Ig isotypes induced following immunization with phthalate. However, in the adjuvanted groups, there was a clear indication of isotype switching, the most discernible one being IgG2a. In the absence of any adjuvant, phthalate-KLH conjugate could only induce a modest IgG1 sub-classes. All groups significantly induced major IgG sub-classes with the following trend of magnitude IgG1> IgG2b≥IgG3≥ IgG2a. Interestingly, PHIS-01 treated group developed a significant IgG2a and IgG2b compared to all other treated adjuvant group. While the isotype profile of anti-phthalate response isotype was marked by increase in IgG1 subclass, the anti-DNA response induced consisted mostly of IgM class. The level of IgG isotype was low and followed a different trend in magnitude as seen with phthalate specific response (IgG2a> IgG1≥IgG3>IgG2b).

Chemokine Profile

Chemokines are considered important regulators of innate immunity response. It is very likely that adjuvant efficacy lies in their ability to induce chemotactic factors and pro-inflammatory cytokines, which regulate the interplay and cross-talk between innate and acquired immunity systems. As shown in Figure 31 and analyzed in Figure 32, treatments with phthalate KLH alone or in combination with different adjuvants resulted in induction of clusters

of chemotactic factors, which are known to be responsible for recruitments of cells belonging to innate immunity, namely, neutrophils, monocytes, macrophages and B1 cells. Analyses of Figure 32A show that LIX and MIP-γ (high expression,) MCP-1, lymphotactin, SDF-1, MCSF, Eotaxin, Eotaxin 2, KC, I-TAC, and MIG (medium express) were induced by antigen alone and also in combination with ALUM, PHIS-01, PHIS-03. SIS-treated groups expressed the same cytokine but less pronounced. Interestingly, PHIS-01 induced more BLC, GCSF and FAS ligand. Later after 5 month, when all adjuvanted and control mice groups received phthalate KLH alone, the chemokine profiles (Figure 32B) differed among groups in term of magnitudes. Mice receiving only phthalate-KLH regimen expressed only LIX and MIP-1γ, which seem to be constitutive in NZB/WF1 mice. Whereas PHIS-01 and PHIS-03, SIS-H and SIS-M down- regulated the expression of MIG and TIMP-1, alum augmented expressions of I-TAC, G-CSF, Eotaxin, MIG, lymphtactin and MCP-1 (Figure 32). Interestingly, mice receiving SIS-H and SIS-M both up-regulated the expression of BLC suggesting an increase in B1 cells involvement.

Cytokine Profile

Cytokines play an important role in the initiation and progress of distinct and selective of immune response to antigenic stimulation. Based on cytokine production, two extreme spectrum of immune responses have been described Th1-like and Th-2 like responses. The Th1- like response is characterized by production of cytokine IFN-γ, IL-2, IL-12, and G-CSF that stimulate strong cellular immune responses. On the other, in Th2 response induces cytokines such as IL-3, -4, -5, -6, -10, and IL-13. However, an imbalance between Th1 and Th2 cytokine has been shown to be a hallmark of lupus [243]. In order to assess the cytokine

profile induced in immunized mice, we collected sera after 2nd booster immunizations from groups treated only with phthalate-KLH alone or in combination with adjuvants.

The cytokines profiles are summarized in Figure 31 and analyzed in Figure 33. As shown in Figure 33A, NZB/WF1 mice immunized with phthalate KLH alone or with adjuvants registered moderate expressions of cytokines that modulate both Th1(IFN-γ, IL-12) and Th2 (IL-3, -4, -10, and -13) population, and a low but detectable levels of pro-inflammatory cytokine associated with inflammatory response (IL-1, IL-6, IL-17, TNF-α). Alum and PHIS-01 produced an upward trend in IL-1β, IL-17, IL-6, and IL-10, while PHIS-03 induced more IL-12 P70. All adjuvant-treated groups induced high levels of anti-inflammatory cytokines TNFRII, TNFRI, TIMP1. They also had a significantly higher level of IL-4 compared to INF-γ which may suggest Th2 dominance. Expression of cytokines described above was less pronounced in groups treated with SIS-H and SIS-M.

Interestingly, five month after 2nd booster immunization, NZB/WF1 mice that received only Phthalate KLH had significantly lower expressions of cytokine than those in adjuvanted groups. This was notwithstanding the difference among adjuvanted groups. Clearly, adjuvants in vaccine formulation made a difference. Adjuvants not only magnified antibody response but also increased over-all of the encounter with the antigen. This better memory induction conferred by adjuvants use was due to changes in chemokine/cytokine microenvironment. During this stage of 32-week post-immunization, PHIS-03 induced increased expression of IL-1β, INF-γ, IL-3, IL-17 and TNFα to similar level registered by mice immunized PHIS-01 or alum. No significant change was noticed on the level of these cytokine in mice treated either with SIS-H or SIS-M preparations. It is noteworthy that PHIS-03 and SIS-M remarkably down-regulated the levels of TNFR I, TNFR II, and IL-12 P40P70, whereas SIS-H, alum and

PHIS-01 had maintained these cytokine factors as they were after the 2nd booster injection (at 12 week of age). All adjuvant-treated groups, however, remarkably down-regulated the expression Of TIMP-1.

Signs of Nephritis

Repeated immunization of NZB/W F1 mice with phthalate-KLH plus different adjuvants resulted in significantly higher anti-phthalate response and ominously anti-DNA response among groups treated with alum, SIS-H, or PHIS-01. In order to determine whether any clinical signs of nephritis were evident by development of phthalate-induced anti-DNA antibody, we determined urinary protein and blood urea nitrogen (BUN) levels at 8 months of age in all NZB/WF1 groups. Results in Table 8 revealed that mice treated with phthalate-KLH plus alum or SIS-H had higher levels of anti-DNA than in PHIS-01, PHIS-03 or SIS-M. On examination of kidney tissues for histopathological changes in a double blind fashion, it has been apparent that there was no major change in connective tissues or glomerular abnormalities between untreated or adjuvant-treated groups (Figure 34). We primarily detected different level of lymphoid infiltration. Group treated with alum, PHIS-01, PHIS-03, and SIS-H had medium lymphoid aggregates. Whereas Kidneys from SIS-M as well as antigen alone groups registered small lymphoid infiltration.

These results suggest that while treatments with Phthalate-KLH in combination with adjuvant tested induced increased anti-DNA levels, no severe sign of severe nephritis or abnormality in kidney tissue were observed. Furthermore, mice did not die prematurely in all adjuvant-treated groups except in alum-treated group where 2 out 5 mice treated died at 8 month of age, and only 1 mouse out of 5 died in group of mice treated with SIS -H or PHIS -01 group or not treated mice. However, there was no mortality in mice treated only with

phthalate-KLH or in combination with PHIS-03or SIS-M. The normal life span of NZB/WF1 mice is between 8 and12 months.

Discussion

The objective of this study has been to assess whether new Phytol-based adjuvants, PHIS-01 and PHIS-03, and ECM-derived SIS adjuvants are comparable, if not superior to alum, the standard adjuvant in autoimmune prone in NZB/WF1 mice that served as an animal model for the study of systemic lupus erythematosus (SLE). As an antigen for vaccination, we used phthalate-KLH, an environmental hazard that can aggravate autoimmune responses in these mice [142]. Previously, we showed that phthalate as a conjugate or as DEHP (diethyl hexyl phthalate, a plasticizer) can induce cross reactive anti-DNA antibody response, and promote lupus-like syndromes in NZB/WF1 mice [142, 170, 244]. The ability of phthalate to induce both anti-phthalate and cross reactive anti-DNA antibodies was, therefore, utilized to assess the efficacy and safety of novel adjuvants, PHIS-01, PHIS-03, SIS-H and SIS-M relative to alum.

Our results show that phytol-based adjuvants and SIS biomaterials are as effective as alum in enhancing anti-phthalate antibody response. However, their impacts differed in respect of cross reactive anti-ds-DNA response as these mice got older. In these immunized mice (3-month old) response to phthalate has been enhanced production of anti-phthalate antibody in adjuvanted groups. Relatively, anti-ds-DNA response is low in PHIS-03 and SIS-M compared to that in alum, SIS-H, and PHIS-01. Moreover, anti-DNA response is of low titer, low-affinity IgM type antibody. Only high affinity IgG2a and IgG3 antibody classes and not IgM are considered pathogenic that aggravate the lupus-like diseases [135, 245]. Furthermore, previous reports have demonstrated that IgM autoantibodies induced as part of an autoimmune response

may actually reduce the severity of autoimmune pathology associated with IgG autoantibodies [245]. However, at ages of eighth months, we can measure Ig2a and other isotypes, particularly in antigen-treated and alum groups. The experimental adjuvants are superior to alum in this regard.

Assessment of cytokine/ chemokine supports the aforementioned above finding. During this study, we have hypothesized that appropriate adjuvants can alter host microenvironment specifically the cytokine milieu; this may bestow ameliorating effects by changing the course of immune response. Cytokines and chemokines play an essential role in outcome of immune response either promoting a productive immune response with or without adverse effects[89]. The profiles of chemokines, proinflammatory cytokines, and Th1/ Th2 ratios assessed in this study clearly establish that PHIS and SIS compounds have ameliorating effects as adjuvants. Both alum and new adjuvants are capable of inducing chemokines such as LIX, BLC, MCP-1, RANTES, and Eotaxin. These chemokines are necessary for the recruitment of neutrophils, monocytes, macrophages and immature dendritic cells (iDc), as well as B cells; all play important roles in uptake of antigen and subsequent development of adaptive response [194]. However, over expression of chemokines such as MCP-1, RANTES, or BLC has been linked to lupus nephritis in patients and in animal models of the disease [246-250]. But at 8 months of age and despite repeated exposure to phthalate, all adjuvanted groups promote only moderate levels of these chemokines.

Overall, only limited pro-inflammatory response has been observed in all groups. The pro-inflammatory response in term of IL-1α, IL-1β, Il-6 and TNF-α due to PHIS-01 and PHIS-03 is similar to what we observe with alum. SIS-H and SIS-M biomaterials are even better; they provoke no measurable pro-inflammatory cytokines. Interestingly, PHIS-01, PHIS-03 and

alum can cause moderate lymphocyte infiltration as shown in kidney histology, in contrast, SIS-treated groups is similar to non-adjuvant group, only caused small infiltration.

Infiltration of neutrophils and monocytes to kidney is known to correlate with proteinuria and onset of kidney damage [89, 251]. There is virtually no onset of renal pathology in mice that are 8-month old. This is unlike what has been reported on Freunds' adjuvants and squalene in MF59 [11, 124, 171, 252]. Possibly the explanation lies in IL-10, which actually goes up upsetting pro-inflammatory forces. Alum and PHIS-01 cause marked increase in IL-10 expression balancing the effect caused by pro-inflammatory cytokines. However, this is not the only reason for the outcome of immune response to phthalate triggered by each adjuvant.

Another hallmark of lupus in the imbalance of Th1/ Th2 profile [243]. In a previous study, it has been shown that hydrocarbon oil adjuvant like pristane can induce an overproduction of Th1 polarizing cytokines like INF-γ [243]. This and high level of IL-6 and TNF-α can aggravate lupus-like diseases in rodents [252]. In our study, the Th1/ Th2 balance as measured by the level of Th1 (IL-4, IL-13) and Th2 cytokine (INF-γ) shortly after 3rd immunization and after 4th immunization with antigen alone does not profoundly change among the adjuvanted groups. However, alum, PHIS-01 and PHIS-03 can induce more Th1 and Th2 cytokines than SIS materials. In addition, PHIS-01 is very effective in inducing IL-12 and is the only adjuvant that can facilitate the production of significant IgG2a subclass signifying a shift towards Th1. Interestingly, the Th1 and Th2 responses generated by different adjuvants is more directed toward phthalate as evident by induction of IgG subclass, whereas the cross reactive ant-ds DNA response was mostly IgM with little IgG subclass switching indicating no affinity maturation or memory indication characteristics of T helper activity on antigen specific

B cells. This ability of adjuvants to selectively activate antigen-specific T cells without provoking auto-reactive T cells is of great interest in adjuvant design. It is worthy to assess also the efficacy of adjuvants at the level of antibody gene repertoire selection, especially at the level of the antibody light chain repertoire. As previously documented in several reports, induction of specific repertoire of light chain-like V kappa1 genes greatly increase the pathogenic properties of autoantibodies produced during autoimmune response[253, 254]. Our ongoing study would focus on characterization of antibody light chain repertoire induced by different adjuvants and its significance on suppression or aggravation of phthalate induced lupus like autoimmune response.

Figure 29.

Immunization of Autoimmune NZB/W F1 Mice with Phthalate-KLH Conjugate Induces both Anti-phthalate Antibody and Anti-DNA Response.

The results represent average of 5 mice sera tested individually using ELISA. A) Anti-phthalate antibody levels after 2nd booster immunization. B) Anti-DNA antibody levels after 2nd booster immunization. C) Anti-phthalate antibody levels after 3nd booster immunization with antigen alone. D. anti-DNA antibody levels after 3nd booster immunization with antigen alone.

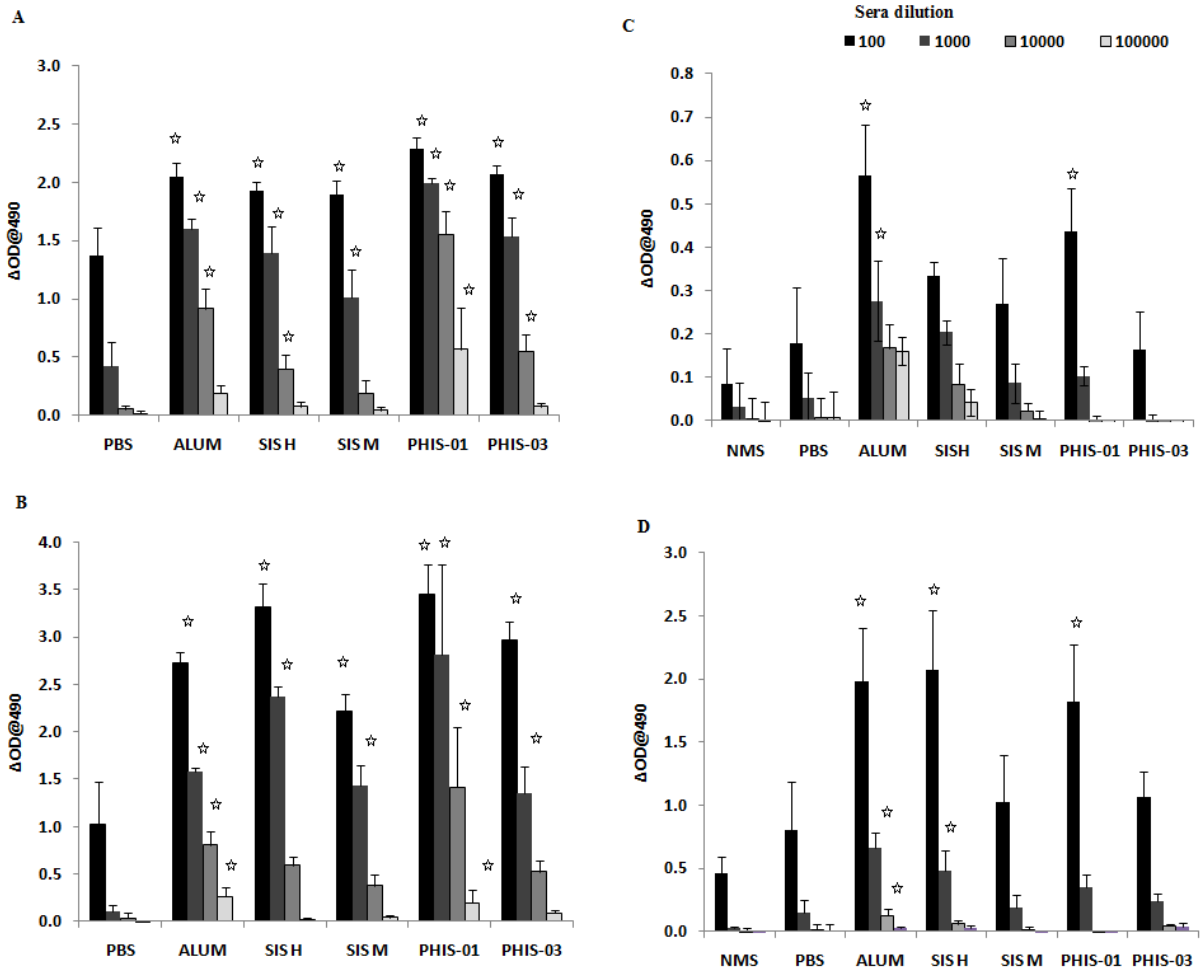
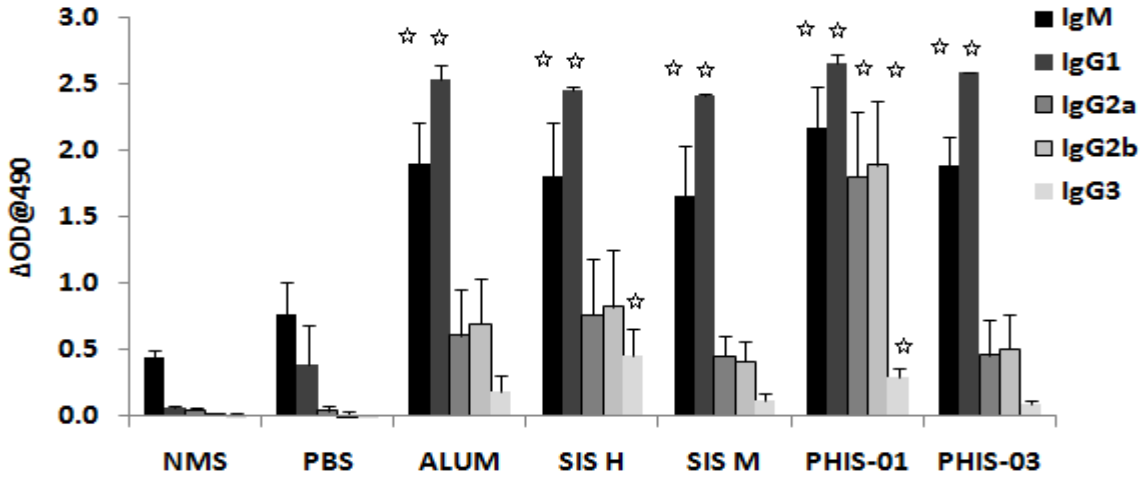


Figure 30.

Determination of IgG Sub-classes.

Determination of IgG sub-classes of (A) Anti-phthalate antibodies and (B) Anti-DNA induced with phthalate-KLH conjugates in different adjuvants. The results represent average of 5 mice sera tested individually using ELISA using commercial ELISA isotyping kits.

A



B

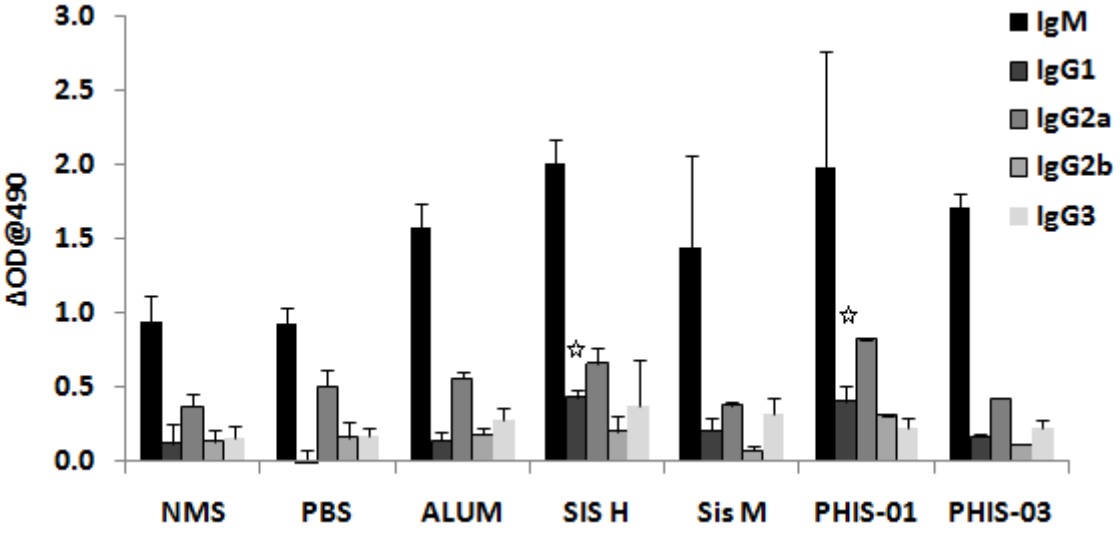


Figure 31.

Systemic Cytokine and Chemokines Profile of NZB/W F1 Mice Immunized with Phthalate-KLH.

NZB/W F1 mice (N=5) were immunized as described in material and methods, and sera collected after 2nd booster, and 3rd booster with antigen alone were diluted 1:5 and subjected to cytokine and chemokines antibody arrays. Each cytokine is represented by duplicate spots in the array as shown in the array template. Image shown in (A) represents cytokines and chemokines profile of mice treated with different adjuvants in combination with antigen after receiving two booster immunizations, and image shown in (B) represents cytokines and chemokines profile of mice treated with different adjuvants in combination with antigen after receiving a 3rd booster immunization with antigen alone.

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	Blank	BLC	CD30 L	Eotaxin	Eotaxin-2	Fas Ligand	Fractalkine	GCSF
2	POS	POS	NEG	NEG	Blank	BLC	CD30 L	Eotaxin	Eotaxin-2	Fas Ligand	Fractalkine	GCSF
3	GM-CSF	IFNγ	IL-1α	IL-1 β	IL-2	IL-3	IL-4	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70
4	GM-CSF	IFNγ	IL-1α	IL-1 β	IL-2	IL-3	IL-4	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70
5	IL-13	IL-17	I-TAC	KC	Leptin	LIX	Lymphotactin	MCP-1	MCSF	MIG	MIP-1α	MIP-1γ
6	IL-13	IL-17	I-TAC	KC	Leptin	LIX	Lymphotactin	MCP-1	MCSF	MIG	MIP-1α	MIP-1γ
7	RANTES	SDF-1	TCA-3	TECK	TIMP-1	TIMP-2	TNFα	sTNF RI	sTNF R II	Blank	Blank	POS
8	RANTES	SDF-1	TCA-3	TECK	TIMP-1	TIMP-2	TNFα	sTNF RI	sTNF R II	Blank	Blank	POS

Figure 31 (Continued).

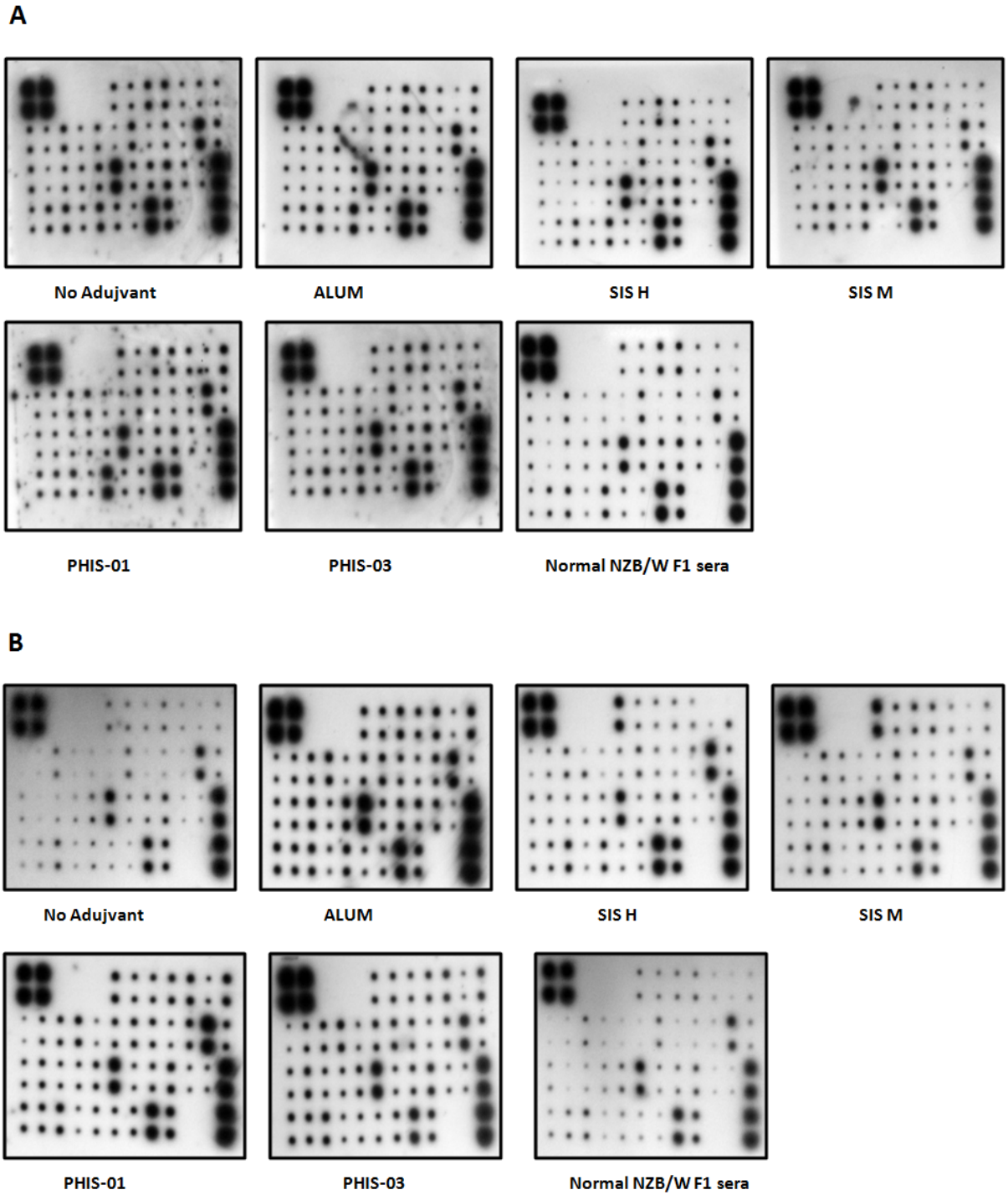


Figure 32.

Systemic Chemokines Profile of NZB/W F1 Mice Immunized with Phthalate-KLH.

NZB/W F1 mice (N=5) were immunized as described in materials and methods, and sera collected after 2nd booster, and 3rd booster with antigen alone were diluted 1:5 and subjected to cytokines and chemokines antibody arrays. Each cytokine is represented by duplicate spots in the array as shown in the array template. Densities of each spot from images shown in figure 3 were semi-quantified using inage j software and expressed as relative units to positive controls provided in the array (A) represent chemokines profile of mice treated with different adjuvants in combination with antigen after receiving two booster immunization, and image shown in (B) represents chemokines profile of mice treated with different adjuvants in combination with antigen after receiving a 3rd booster immunization with antigen alone.

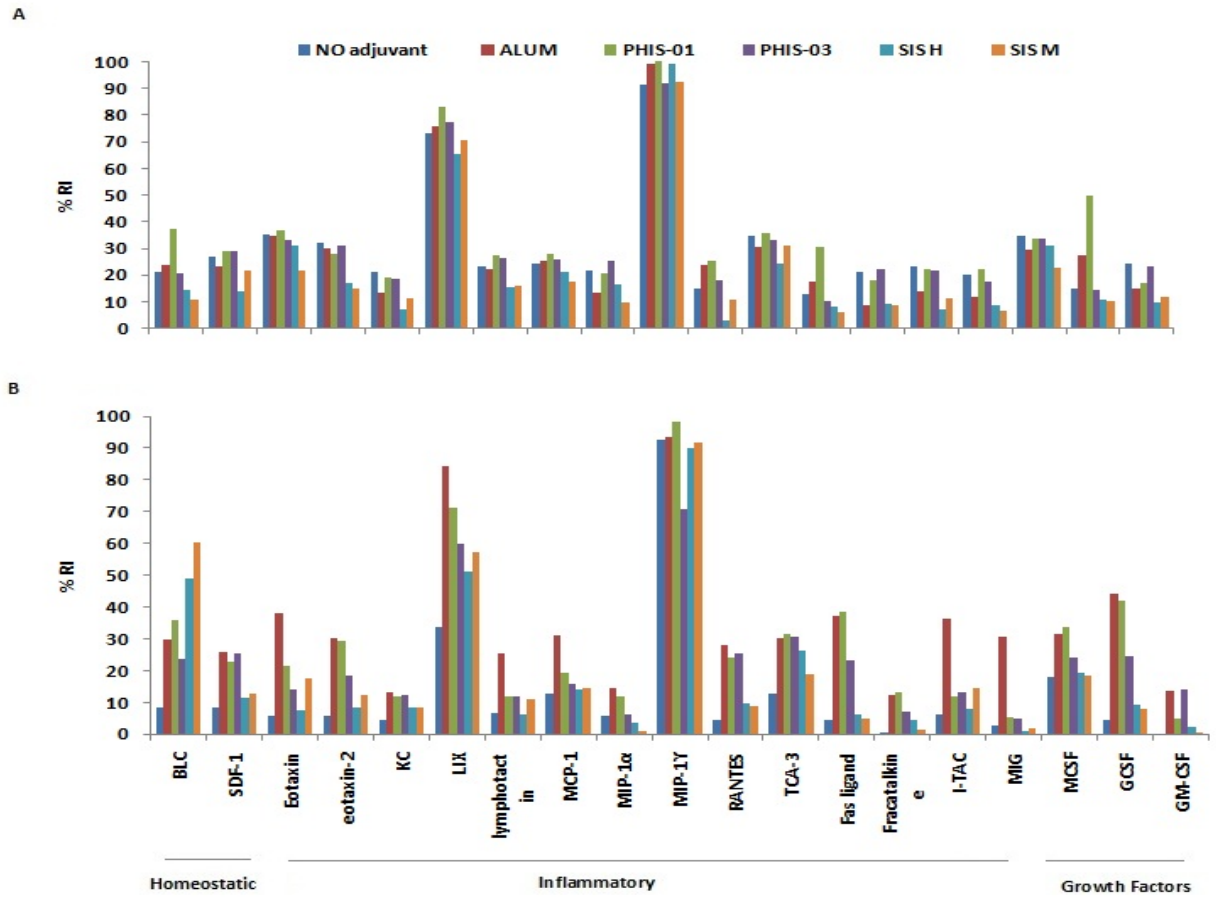


Figure 33.

Systemic Cytokines Profile of NZB/W F1 Mice Immunized with Phthalate-KLH.

NZB/W F1 mice (N=5) were immunized as described in materials and methods, and sera collected after 2nd booster, and 3rd booster with antigen alone were diluted 1:5 and subjected to cytokines and chemokines antibody arrays. Each cytokine is represented by duplicate spots in the array as shown in the array template. Densities of each spot from images shown in figure 3 were semi-quantified using inage j software and expressed as relative units to positive controls provided in the array (A) represents cytokines profile of mice treated with different adjuvants in combination with antigen after receiving two booster immunization, and image shown in (B) represents cytokines profile of mice treated with different adjuvants in combination with antigen after receiving a 3rd booster immunization with antigen alone.

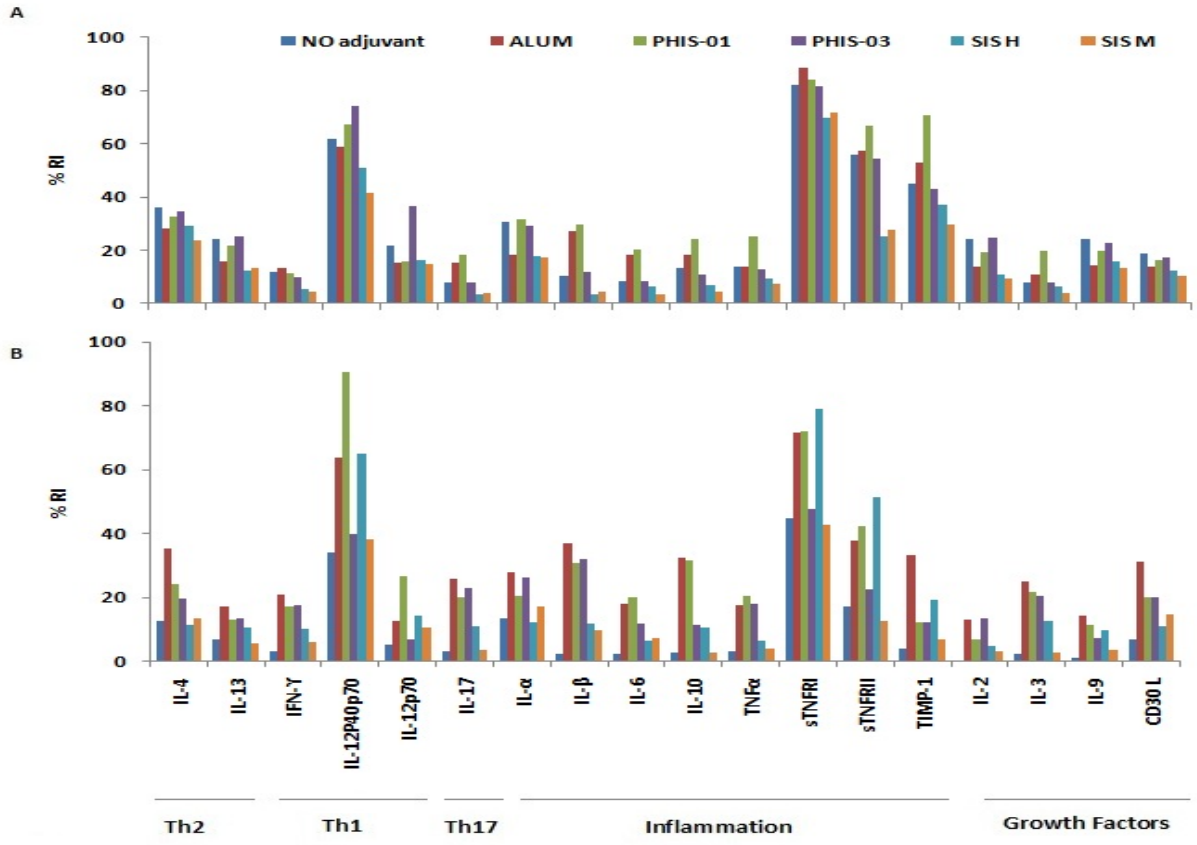


Table 8.

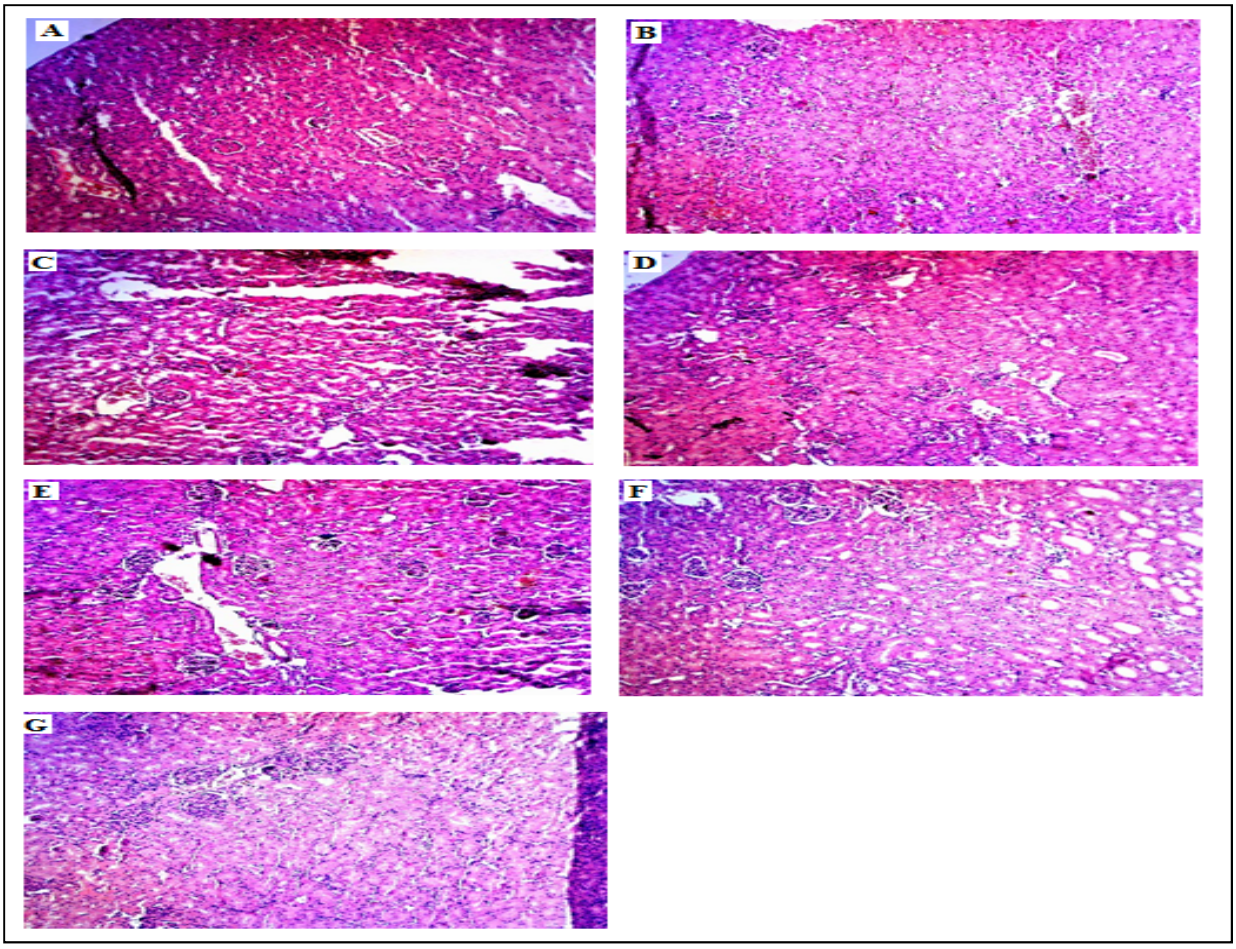
Assessment of Clinical Sign of Kidney Pathology.

	NZB/W F1 immunized with Phthalate- KLH in combination with different adjuvants						
	Untreated mice	No adjuvant	ALUM	PHIS-01	PHIS-03	SIS-H	SIS-M
Proteinura (mg/dL)	0.3	126.0	166.7	132.0	47.5	232.0	82.5
BUN (mg/dL)	15.0	39.6	53.33	46.5	23.25	56.5	33.0

Figure 34.

Renal Histopathology.

Kidney tissues of seven groups of NZB/W F1 mice at 8 months of age were examined after immunization with Phthalate-KLH conjugate emulsified in different adjuvants as described in materials and methods. Representative H&E stained kidneys (magnificationx100) are shown as follow: group A: age matched mice. Group B: Mice immunized with phthalate-KLH alone. Group C: Mice immunized with phthalate-KLH adsorbed to alum. Group D: Mice immunized with phthalate-KLH emulsified with PHIS-01. Group E: Mice immunized with phthalate-KLH emulsified with PHIS-03.Group F: Mice immunized with phthalate-KLH emulsified with SIS-H. Group G: Mice immunized with phthalate-KLH emulsified with SIS-M.



CHAPTER 7

SYNOPSIS

Rationale

Vaccines are considered one of the most successful medical advances that arguably have the greatest benefits on human health over the last two centuries. By inducing high titer neutralizing antibody or cell mediated effectors, accompanied by a long lasting immunological memory, vaccines have effectively prevented and eradicated many life threatening diseases. However, they are largely ineffective without adjuvants. Although, a number of substances are potentially adjuvants, the list of clinically approved adjuvants for human use is limited. It includes primarily two adjuvants: alum adjuvant based on aluminum hydroxide and/or aluminum phosphate, and MF59, which contains the terpenoid squalene an intermediary in cholesterol biosynthesis. A common denominator between these two human licensed adjuvants is that they are deemed safe and immunologically effective. Alum largely promotes an immune response involving T-helper type2 lymphocytes [114], while MF59 promote a balanced Th1/ Th2 immune response [118]. Overall, both work, but not versatile for all vaccines. Alum is implicated in dementia and squalene in arthritis and autoimmunity. To facilitate selection of adjuvants not empirically, but on the basis of clearly delineated modes of actions, the current study focused on correlative structure-function studies and understanding of the basic physico-chemical properties required for adjuvanticity.

Terpenoids as Adjuvants

The quest for defined and broadly effective immunostimulants has directed this investigation to terpenoids that are most ubiquitous in nature and seem to have beneficial effects against cancer [133]. Terpenoids, like squalene mentioned above, have been shown to provide a protective immunity against external threats [118], although some of these compounds such as squalene, pristane, and phytol can be toxic and have arthritogenic and auto-immunogenic activity. To overcome this inadequacy and improve adjuvanticity of phytol, the diterpene from chlorophyll, its double bond was reduced to generate the first phytol-derived immunostimulant phytanol (named PHIS-01) [141, 142]. PHIS-01 proved effective with the ability to induce robust and broadly effective immune responses against both external threats such as environmental bacterial hazards, and internal threats due to cancer or autoimmunogens.

New Derivatives of Phytol as Adjuvants

In this study, we modified –OH group in PHIS-01 by amination producing phytanyl amine (PHIS-02) and by mannosylation producing phytanyl mannose (PHIS-03). The objectives were (1) to assess safety and adjuvanticity of these compounds and (2) to examine how changes at the polar terminus affect adjuvanticity of PHIS-02 and PHIS-03 relative to PHIS-01, SIS (ECM from porcine small intestinal submucosa) and alum in terms of bio-response modifiers and adjuvant-responsive core genes.

The findings support the contention that phytol-derived adjuvants are safe and efficacious as immunostimulants. This is on the basis of their ability to promote effective humoral response, stimulate T cell proliferation, but exhibit no adverse autoimmune anti-DNA response in resistant and susceptible mice strains.

Among the phytol derivatives, PHIS-01 is non-toxic and highly effective at a wider range of concentrations (4- 44mg/mouse). PHIS-02 functions at a much lower concentration (2. 5 mg/mouse), and PHIS-03 works effectively at an intermediate doses (5 mg). At these doses, immunization of different strains of mice (BALB/c, C57BL/6 and NZB/W F1) caused no noticeable physical or behavioral changes in mice, no significant fluctuations in their body weights and no splenomegaly or granuloma formation,

In term of adjuventicity, PHIS-01, PHIS-02, PHIS-03 all significantly augment antibody response of isotype IgG1 and IgG2a and promotes high rates of proliferation of in vivo primed splenocytes, particularly T-lymphocytes, as is evident from secretion of IL-2, the T-cell growth factor. Therefore, inclusion of these adjuvants in vaccines would enhance activate T cells and help secretions of many other cytokines from activated T cells. Using PHIS-01 and PHIS-02, we noted a clear bias towards Th1 response as exemplified by IgG2a and INF-γ levels, whereas the use of PHIS-03 induces IL-4, indicating a shift towards Th2 response.

At the cellular level, adjuvants are normally considered to function by inducing limited local inflammation marked by apoptosis/necrosis of target tissues, which facilitates increased antigen uptake and processing by antigen-presenting cells [172, 174]. In this study, we observed that Phytol-based adjuvants also exert a considerable apoptotic/necrotic effect on immune cells in a concentration-dependent manner. These beneficial effects on immunological process by both mechanisms of cell death are shown to be mediated possibly by danger signals that led to changes in host microenvironment in term of chemokines, cytokines, and growth factors.

To better understand the mechanisms of action of Phytol-based adjuvant and their relative potency when compared with other adjuvants, we performed microarray analysis of

cytokines and chemokines environment at sites of injection. In this study, Phytol-based adjuvants displayed significant effects on bioresponse modifiers. Upon immunization, all phytol-based adjuvants provoked a set of chemokines responsible for recruitments and activation of innate immune cells. Among chemokines activated by phytol-derived adjuvants, we note the presence of neutrophil-activating chemokines KC (CXCL1) and LIX (CXCL5) and detectable expressions of esionophile and activated T cell chemotacitce RANTES (CCL5) and Eotaxin-2 (CCL11). We also observed high expressions of MCP-1 (CCL2) that is known to recruit and activate monocytes [197, 198]. In addition, phytol compounds evoked growth factors M-CSF, GM-CSF and G-CSF, which trigger differentiation of monocytes into macrophages, dendritic cells, and stimulate granulocytes respectively [199-201]. Other chemotactic factors released in response to phytol -based adjuvants are chemo-attractants such as BLC (CXCL13) for B lymphocytes, MIP-1α (CCL-3) and MIP-1γ (CCL9) secreted by macrophages for granulocytes and DCs, and TCA and lympotactin for activated T cells and NK cells. The induction of chemokines by phytol derivatives PHIS-01, PHIS-02, PHIS-03 was accompanied by increased expression of pro/anti-inflammatory cytokines (IL-1, IL-6, sTNFR I& II, TIMP-1) and Th1/ Th2 (INF-y, IL-12/ IL-4, IL-13). Together, these bioresponse modifiers indicate that the phytol compounds initiate and activate both the innate and acquired immunity and leads to T helper polarization and magnitude of antibody response. Although all adjuvants strongly affected the cytokine profiles at sites of injection, there was no obvious correlation between the cytokine profile and type of the response mounted. To further resolve this issue, we intend to assess the systemic cytokine profiles induced by each adjuvant as well as investigate functional and phenotypical characteristics of antigen presenting cells recruited at sites of injection. Nonetheless a major difference observed during this study is that PHIS-01

was the strongest Inducer of cytokines, chemokines, and genes involved in APCs and leukocyte activation and maturation compared to PHIS-02 and PHIS-03.

Comparison between PHIS-01 and PHIS-03 at the level of transcription of inflammatory genes shows that PHIS-01 and PHIS-03, similar to alum, induced a common set of core response gene composed of chemokines and cytokines as described earlier. However, the effects of PHIS-01 could be noted in the innate immunity receptors known as Nod-Like receptors (NLRs). They are present in APCs and involved in different NLRs activated pathways. Among these receptors and pathways, the one well studied is NLRP-3 inflammasome. This inflammasome is considered to be important in the functioning of TLR-independent adjuvants exert their effects. Alum's effectiveness is due to NLRP3 inflammasome since there is diminished immune response in NLRP-3 knockout mice indicating indispensability of NLR receptors [192]. PHIS-03 has moderate effects on NLRs and associated genes; it may be a single mannose at its polar terminus that activates a different set of receptors and immune pathways. Mannose motif often present at the surface of infection agents is known to involve mannose receptors on APCs and activate mannose-mediated lectin pathway for complement activation [255]. Whether this happens or not is not known, and may be pursued in a future study.

Regardless of the mechanisms involved, all Phytol derivatives have suppressive effects on the manifestation autoimmune response caused by phthalate in both non/pro autoimmune mice. While oil-adjuvant like squalene or CFA are proven to exacerbate such response [11, 124, 171, 252], phytol derivatives evoke little anti-DNA antibody response and engender non-aggravating host micro environment.

In separate experiments, we assessed the adjuvanticity of another classes of vaccine adjuvants based on ECM, the SIS material (Cook Bio-Tech). The study of SIS adjuvant was initiated in prospect to further the adjuvanticity of phytol compounds by creating cocktail adjuvants. SIS adjuvants (SIS H and SIS M two separate batches), similar to alum, promote high and ling-lasting IgG1 antibody response mediated by Th2 immune response. In addition, SIS adjuvants neither mobilize innate immunity through chemokines and cytokines like alum, nor involve any pro-inflammatory cytokines or activated NLRP-3 inflammasome. The inclusion of SIS adjuvant in a cocktail containing any phytol derivative may potentiate their immunological effects by complementing their respective immunomodulatory capabilities. Since SIS biomaterials are excellent tissue remodeling agents, this would effectively create a wound healing environment to quickly resolve any tissue injury and inflammatory environment known to be caused by oil-in-water adjuvants.

In conclusion, modifications of polar terminus of PHIS-01 with amine moiety (PHIS-02) or a hydrophilic mannose moiety (PHIS-03) produce interesting bioactive compounds. PHIS-02 is effective at a lower dose, Affects cytokines and chemokines milieu and facilitates T helper polarization (Th1and high antibody response as PHIS-01). Moreover, PHIS-03 works at lower doses; it changes cytokines/chemokines milieu, but polarizes T helper cells towards Th2 and provokes significant antibody response.

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APPENDIX A: SUPPLEMENTAL DATA

Table 1S.

Transcription of inflammasome pathway genes in the mouse peritoneum.

The level of expression is shown as ± Fold change compared with PBS controls sacrificed at the same time point.

Gene	OVA	ALUM	ALUM+OVA	SIS-H	SIS-H+OVA
Ccl12	1.0	2.8	1.6	-1.7	-1.0
Ccl5	-1.8	-1.1	-1.1	-1.0	1.3
Ccl7	1.0	3.6	3.1	-1.5	1.0
Cxcl1	-1.3	1.3	1.2	-1.3	-1.3
Cxcl3	-1.3	3.5	2.0	1.0	1.1
Ifnb1	1.2	1.9	3.3	2.4	2.2
Ifng	-2.0	1.4	2.3	1.6	1.8
Il12a	-1.1	1.1	1.3	1.4	1.2
Il12b	-1.2	3.4	5.0	2.3	2.1
Il18	1.1	-1.2	1.1	1.5	1.4
Il1b	-1.3	4.4	3.8	1.7	1.6
Il33	1.1	1.6	2.5	1.4	1.2
Il6	-1.1	-1.4	-1.2	-1.1	-1.1
Cd40lg	-1.2	-1.3	1.4	1.4	-1.1

Table 1S (Continued).

Gene	OVA	ALUM	ALUM+OVA	SIS-H	SIS-H+OVA
Tnf	1.2	3.8	3.0	1.3	1.2
Tnfsf11	-1.2	-1.1	2.5	1.5	1.3
Tnfsf14	-2.0	1.9	2.6	-1.0	1.4
Tnfsf4	1.5	1.0	1.6	1.7	1.5
Aim2	-1.3	-1.8	-1.1	-1.2	1.1
Bcl2	-1.3	-2.3	-1.6	-1.3	1.0
Bcl2l1	1.0	1.6	1.3	-1.4	-1.2
Birc2	-1.0	-1.2	1.0	-1.1	-1.0
Birc3	-1.0	1.4	1.4	1.0	1.1
Card6	-1.4	-1.2	-1.1	-1.2	-1.0
Casp1	-1.0	1.0	1.1	-1.2	1.3
Casp12	1.4	1.3	3.3	1.7	2.8
Casp8	-1.3	-1.9	-1.2	-1.2	1.0
Cflar	-1.1	1.4	1.4	-1.0	-1.1
Ctsb	1.2	1.3	1.5	1.1	1.1
Fadd	-1.1	-1.4	1.2	-1.0	-1.2
Naip1	-1.3	-2.8	-1.5	-1.4	-1.1
Naip5	-1.2	-1.7	-1.1	-1.3	1.0
Nlrc4	-1.3	-2.0	-1.5	-1.2	1.0
Nlrc5	-1.4	-1.4	-1.2	-1.7	-1.2
Nlrp12	-4.3	1.8	2.0	-1.5	-1.0

Table 1S (Continued).

Gene	OVA	ALUM	ALUM+OVA	SIS-H	SIS-H+OVA
Nlrp1a	-1.1	-1.2	1.3	-1.5	-1.2
Nlrp3	-1.4	1.6	1.8	-1.3	1.4
Nlrp4b	-1.1	1.0	3.6	1.5	3.1
Nlrp4e	-1.1	-1.1	2.4	1.3	2.2
Nlrp5	-1.1	1.2	2.9	1.8	2.7
Nlrp6	1.2	-1.2	2.8	1.2	1.6
Nlrp9b	1.3	2.1	4.3	2.0	3.3
Nlrx1	1.1	1.5	1.5	-1.3	1.1
Nod2	-1.2	1.5	1.4	-1.4	1.1
P2rx7	-1.4	-1.1	-1.1	-1.2	1.0
Panx1	1.0	-1.3	-1.0	-1.1	1.4
Pea15a	1.1	-1.6	-1.0	-1.1	1.3
Pstpip1	-1.1	1.7	1.6	-1.1	1.4
Ptgs2	-1.1	3.6	3.8	1.3	1.8
Pycard	-1.1	-1.3	-1.1	-1.5	-1.0
Txnip	1.0	1.5	1.7	1.1	1.4
Xiap	-1.0	1.1	1.2	1.1	1.0
Mefv	-1.1	3.3	2.7	1.2	1.3
Chuk	-1.2	-1.6	1.0	1.0	1.0
Ciita	1.1	1.0	1.2	1.2	1.1
Ikbkb	-1.0	-1.4	-1.1	1.1	-1.1

Table 1S (Continued).

Gene	OVA	ALUM	ALUM+OVA	SIS-H	SIS-H+OVA
Ikbkg	1.1	1.2	1.3	1.1	-1.2
Irak1	1.0	-1.5	-1.2	1.1	-1.2
Irf1	-1.0	1.6	1.4	1.2	1.2
Irf2	-1.1	-1.1	1.0	1.0	1.1
Irf3	-1.2	-1.3	1.3	-1.3	1.1
Map3k7	-1.0	-1.4	-1.1	-1.0	1.1
Map3k7ip1	1.0	-1.1	1.2	-1.3	-1.2
Map3k7ip2	-1.2	1.0	1.1	-1.1	-1.0
Mapk1	-1.1	-1.2	-1.1	-1.2	-1.0
Mapk11	-1.1	-1.9	-1.4	-1.0	1.1
Mapk12	-1.0	-2.0	-1.1	-1.1	1.1
Mapk13	-1.8	1.9	1.8	-1.2	1.0
Mapk3	1.0	1.5	1.3	-1.0	-1.0
Mapk8	-1.2	-1.6	1.1	1.0	1.1
Mapk9	-1.1	-1.4	1.1	-1.0	1.0
Myd88	1.2	1.8	1.4	-1.1	-1.1
Nfkb1	-1.1	-1.0	-1.1	-1.2	1.1
Nfkbia	-1.1	1.6	1.3	-1.2	1.1
Nfkbib	-1.1	-1.0	1.4	-1.2	1.2
Rage	-1.8	-1.8	1.5	-1.1	1.1
Rela	-1.0	1.2	1.1	-1.0	-1.0

Table 1S (Continued).

Gene	OVA	ALUM	ALUM+OVA	SIS-H	SIS-H+OVA
Ripk2	-1.1	-1.4	-1.2	-1.1	1.0
Tirap	-1.2	1.0	1.3	-1.1	1.0