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Tiffany Ding April 14, 2016

Understanding the Impact of LPS and GLA on the Induction of Proinflammatory Cytokines

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract
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Vaccines play a crucial role in the elimination of infectious disease by generating long-term protection against pathogens. Immunostimulatory adjuvants have currently been used in combination with vaccines to further enhance the body's immune response following infection. Toll-like receptor 4 (TLR4) agonist bacterial lipopolysaccharide (LPS) from Gram-negative bacteria is a potent adjuvant responsible for triggering an inflammatory response that results in the production of critical proinflammatory cytokines. Excessive release of these cytokines results in endotoxic shock in animals as characterized by systemic inflammation, organ failure and death. Monophosphoryl lipid A (MPL) is a low-toxicity derivative of LPS that is in development as a vaccine adjuvant due to its ability to retain the immunostimulatory properties of its parent molecule without the endotoxic effects associated with LPS. It is widely recognized that LPS is mediated by TLR4 to initiate two principle signaling pathways: MyD88-dependent and MyD88-independent (TRIF-dependent). MyD88-dependent signaling leads to the induction of proinflammatory cytokines while TRIF-dependent signaling activates Type 1 Interferons. Previous studies propose that TLR4 agonist MPL predominantly activates the TRIF-dependent pathway, a distinction that may be key to MPL's reduced toxicity. Thus, the present study investigates the affects of LPS and a synthetic form of MPL, Glucopyranosyl Lipid A (GLA), on the expression of critical proinflammatory cytokines in murine models using bone marrow derived macrophages *in vitro*. Stimulation with LPS results in increased cytokine expression in comparison to GLA over a time-dependent and concentration dependent response. In addition, cell death regulator Caspase-8 (Casp8) has been implicated as a critical factor in cytokine production downstream of TLR4. Our results reveal that gene expression is substantially reduced in *Casp8*$^{-/-}$*RIP3*$^{-/-}$ mice compared to wild-type mice *in vivo* following LPS or GLA challenge. This data suggests that Casp8 is indispensable downstream of TLR4 receptor activation and an essential hose protease to be studied in vaccine development. Our results provide insight into TLR4 mediated gene expression by GLA that helps define the molecule as an ideal candidate in the design of vaccine adjuvants.

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INTRODUCTION

Background

Disease prevention is key to public health and for the past 100 years, immunizations have played a crucial role in the eradication of and protection against infectious diseases. Vaccines are biological preparations used to produce immunity from harmful pathogens and have become a safe and effective means of generating long-lasting and protective immunity against subsequent infection. The administration of a vaccine mimics a natural infection in a host and stimulates the immune system to recognize, destroy and remember the foreign invader so that the immune system can produce a stronger response against future exposure. Traditional vaccines can be subdivided into several major categories including live attenuated vaccines, killed (inactivated) whole cell vaccines and subunit vaccines. Live attenuated vaccines typically contain an agent from a weakened microorganism and produce long term immunity with only one or two doses. Most effective vaccines are based on attenuated pathogens but one concern that remains is their ability to revert back to its virulent form. An alternative to live attenuated vaccines is the use of material from killed or inactivated microbes because they are unable to replicate and cannot mutate into a form capable of causing disease. However, these provide a shorter length of protection and a weaker immune response and thus, additional boosters are required to create long-term immunity. Subunit vaccines normally use only the antigenic portion of a pathogen but elicit a less strong immune response. Current vaccine design focuses on the use of highly purified protein components of infectious organisms to produce a more targeted immune response while eliminating the risks and limitations associated with live attenuated or inactivated vaccines. However, vaccines composed of purified peptides lack the features of the original microbe and

produce a weaker immune response. Therefore, additional substances have been added to vaccines to intensify the host's immune response. Such compounds are termed immunologic adjuvants.

Immunostimulatory adjuvants are compounds that greatly enhance the host's immune response by working in conjunction with non-living vaccines. They increase the immunogenicity of weak antigens and increase the speed and duration of the immune response for longer lasting protection. The presence of adjuvants also decreases the dose of antigen required and eliminates the need for additional booster shots. Although adjuvants have been used in vaccines for the past century, few have been approved for use in humans. Aluminum salts in the form of aluminum hydroxide, aluminum phosphate or alum have been the predominant components used in licensed human vaccines such as hepatitis A and B, human papillomavirus and diphtheria-tetanus-pertussis (9, 32, 33, 34). Recently, monophosphoryl lipid A (MPL) has been in development as a vaccine adjuvant due to its immunostimulatory properties and ability to induce the production of cytokines and chemokines, key inflammatory mediators in the innate immune response (12, 17, 32, 34, 35). MPL is already licensed for use in clinical settings as a component of the HPV vaccine, Cervarix, and the Hepatitis B vaccine, Fendrix (12). MPL is an attractive candidate as a vaccine adjuvant because of its activation of antigen-presenting cells crucial for the interaction between innate and adaptive immunity (18, 32, 34, 36). The adaptive immune response is mediated by B cells and T cells, specifically activated T helper cells, Th1 and Th2 cells (16). Th2 cells drive a Type 2 response by activating mast cells, eosinophils and basophils critical in clearing extracellular pathogens. Th1 cells drive a Type 1 response by activating antigen- presenting cells to fight against intracellular pathogens such as viruses and bacteria (16, 17). Alum based adjuvants, in particular, are known to produce a strong antiparasitic Th2 response. However, vaccines against some of the most major causes of death worldwide including malaria and HIV also require a Type

1 response. Effective vaccines would essentially provide a strong B-cell and T-cell response, specifically a Th1-mediated response. Hence, adjuvants that promote a strong Th1 response are being targeted for use in human vaccines.

Immune System Overview

The role of the immune system is to prevent or limit infection and protect against potentially damaging foreign bodies. The cells, tissues and organs of the immune system form a complex network that functions to recognize and defend the body against infectious microbes. The immune system can be divided into two major categories: the innate immune system and the adaptive immune system. The innate immune system provides immediate, nonspecific protection against pathogens and acts as the first line of defense upon exposure to infectious organisms. Generally, the innate defense consists of physical barriers such as the skin and mucous membranes, leukocytes (granulocytes, monocytes, macrophages), natural killer cells and antimicrobial proteins. In contrast, the adaptive or acquired immune system is composed of highly specialized cells, B-cells and T-cells, that proliferate in response to antigens and provides longer lasting protection. Adaptive immunity is developed over time through exposure to specific pathogens to produce immunological memory.

Both the innate and adaptive immune response are dependent on the activities of the white blood cells or leukocytes that originate in the bone marrow. The hematopoietic stem cells of the bone marrow give rise to the different red blood cells and the two main subdivisions of white blood cells, the myeloid and lymphoid lineages. The myeloid lineage is the precursor to the macrophages, granulocytes (neutrophils, basophils, eosinophils), mast cells and dendritic cells of the innate immune system. The lymphoid lineage comprises the natural killer cells and the two

types of lymphocytes, B lymphocytes (B-cells) and T lymphocytes (T-cells), of the adaptive immune system.

When an infection is detected, the innate immune system provides an immediate response via inflammation and promotion of antigen presenting cells (APCs). These cells of the innate immune system recognize the molecular structures present on microbes and foreign entities referred to as pathogen-associated molecular patterns (PAMPs) by means of pattern recognition receptors (PRRs) (9, 43). Macrophages, neutrophils and dendritic cells present these PRRs and bind to the common molecular patterns of pathogens (43). Upon engagement of the receptors, cells such as macrophages localized in the tissue are triggered and inflammatory mediators are released (45). The release of chemokines and cytokines by activated macrophages then stimulates the process known as inflammation (45). Cytokines are important regulators of the host's immune response with some acting as pro-inflammatory cytokines to promote inflammation and others as anti-inflammatory cytokines to suppress inflammation (46). Pro-inflammatory cytokines include Interleukin (IL)-1, Interferons and tumor necrosis factor (TNF) and are induced by infection or toxins to initiate an inflammatory cascade (46). The release of an excessive amount of proinflammatory cytokines is detrimental to the host and tends to worsen a disease by promoting fever, system inflammation and shock. Inflammation is typically characterized by vasodilation and the accumulation of fluid at the site of infection, which causes heat, redness and swelling. In addition, the increased vascular permeability leads to the movement of fluid and proteins into the tissues (45). Cytokines produced by macrophages and dendritic cells facilitate the migration of neutrophils and other phagocytic cells to the infected region. The increase in the flow of antigen-presenting cells into the lymphoid tissue contributes to the activation and regulation of the adaptive immune response.

Adaptive immunity is initiated when an infection overwhelms the innate immune response (50). Unlike the innate immune response, the adaptive immune response is highly specific to a particular pathogen and is often referred to as an antigen-specific immune response. Adaptive immunity protects the body against reinfection by creating immunological memory after an initial response to that specific antigen. It learns, adapts and remembers the antigen upon first exposure, which leads to an enhanced immune response upon subsequent encounters with the pathogen. The cells that mediate the adaptive immune response consists of the lymphocytes, B-cells and T-cells. Both lymphocytes display either B-cell receptors (BCRs) or T-cell receptors (TCRs) that bind to specific antigens in the body in order to generate a targeted response. B cells proliferate into plasma cells to secrete antibodies that are specific to the antigen, which causes them to become easier targets for destruction by phagocytes and other immune cells. In response to pathogens, different types of T cells can be activated in the body. Cytotoxic T-cells (CD8+ T-cells) attack and destroy infected or damaged cells while Helper T cells (CD4+ T-cells) support the activity of other immune cells. Helper T cells can be further subdivided into two major subtypes, Th1 helper cells and Th2 helper cells. Th1 helper cells primarily produce interferon-gamma (IFN-γ) and interleukin-2 (IL-2) and stimulates a cell-mediated immune response which involves the promotion of phagocytes and cytotoxic T-cells. On the other hand, Th2 helper cells secrete IL-4, IL-5, IL-6 IL-10 and IL-13 and generate a humoral immune response that is mediated by the antibodies produced by plasma cells. Although most B-cells and T-cells die off after performing their primary functions, some of the remaining lymphocytes can develop into memory cells. Memory cells remember the antigen upon first exposure and generate a more rapid and vigorous immune response after future encounters.

Toll-Like Receptor 4 (TLR-4) Ligands

Collaboration of the innate and adaptive immune system during infection to eliminate microbes from the body is dependent on different cells and their ability to recognize specific pathogenic features. In particular, antigen presenting cells (APCs) play a key role in the immune response because of their presence in all tissues of the host and their ability to elicit a specific immunological response. APCs such as macrophages or dendritic cells are activated upon identification of certain ligands called Pathogen-Associated Molecular Patterns (PAMPs) which are distinct molecules associated with various pathogens (9, 31). They are recognized by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), transmembrane proteins expressed by a variety of cells that are crucial in mediating the innate and adaptive immune response (43). TLRs recognize invading pathogens and activate signaling pathways to produce an immune response (42). Activation of macrophages occurs when a pattern recognition receptor binds to distinct TLR ligands such as bacterial cell-wall components and double-stranded RNA (dsRNA) and double-stranded DNA (dsDNA) (43, 50). When a TLR is activated by PAMPs, a signal is triggered that induces macrophages to synthesize various chemokines and cytokines and stimulate the adaptive immune response (9, 42, 50).

In hosts, TLR4 is considered an important element in the triggering of innate immunity following bacterial infection. Specifically, TLR4 recognizes pathogen associated molecule bacterial lipopolysaccharide (LPS), the primary cell wall constituent in Gram-negative bacteria. Bacterial LPS is the major endotoxin component of the outer membrane of Gram-negative bacteria and consists of a hydrophobic lipid A, a hydrophilic core polysaccharide chain and O-antigen repeating units. The lipid A portion of the molecule is responsible for exerting the bacteria's endotoxic effects. Lipid A stimulates macrophages via TLR4 to trigger the secretion of

proinflammatory cytokines which can lead to the pathogenesis of septic shock that results in organ failure, systemic inflammation and ultimately, death (4, 50). Upon LPS recognition, TLR4 triggers recruitment of downstream adaptor molecules, myeloid differentiation factor 88 (MyD88) and TRIF, to initiate two signaling pathways: MyD88-dependent and MyD88-independent (TRIF-dependent) (3, 24). The MyD88-dependent TLR4 signaling cascade uses adaptor protein MyD88 to recruit and activate IL-1 receptor associated kinases (IRAKs). The kinase associates with adaptor protein TNF receptor-associated factor 6 (TRAF6) and forms a complex that leads to the activation of nuclear factor kappa B (NF-κB) via TANK-binding kinase 1 (TBK1) and the expression of critical proinflammatory cytokines and chemokines such as tumor necrosis factor- α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) (3, 4, 25). Alternatively, the TRIF-dependent pathway is mediated by adaptor proteins (TIR-domain-containing adaptor-inducing interferon-β (TRIF) and (TNF Receptor-Associated Factor 3) TRAF3 to activate interferon (IFN)-regulatory factor (IRF3) and the induction of Type 1 Interferon, IFN-β among other interferon stimulated genes (Fig. 1). The ability of LPS to induce the production of pro inflammatory cytokines vital in the immune response establishes the molecule as a valuable vaccine adjuvant (32). Although the bacteria-derived molecule possesses strong immunostimulatory properties, its toxicity prevents it from being used in clinical settings. However, the inflammatory properties associated with the lipid A portion of LPS and the production of cytokines and chemokines following TLR4 stimulation can be used in vaccine development. To further understand the endotoxicity of lipid A, the structure has become a valuable component in the design of various synthetic derivatives. Variation in the phosphorylation state of lipid A as well as differences in the length and number of carbon chains on the molecule have been observed to significantly impact the molecule's toxicity. In particular, it appears that the diphosphorylated form of lipid A such as

diphosphoryl lipid A from *Escherichia coli* Lipid A retains the full endotoxicity of the molecule whereas monophosporyl lipid A is nontoxic (48, 49). Hence, monophosphoryl lipid A has been recognized to be a critical component in the development of adjuvants in clinical settings.

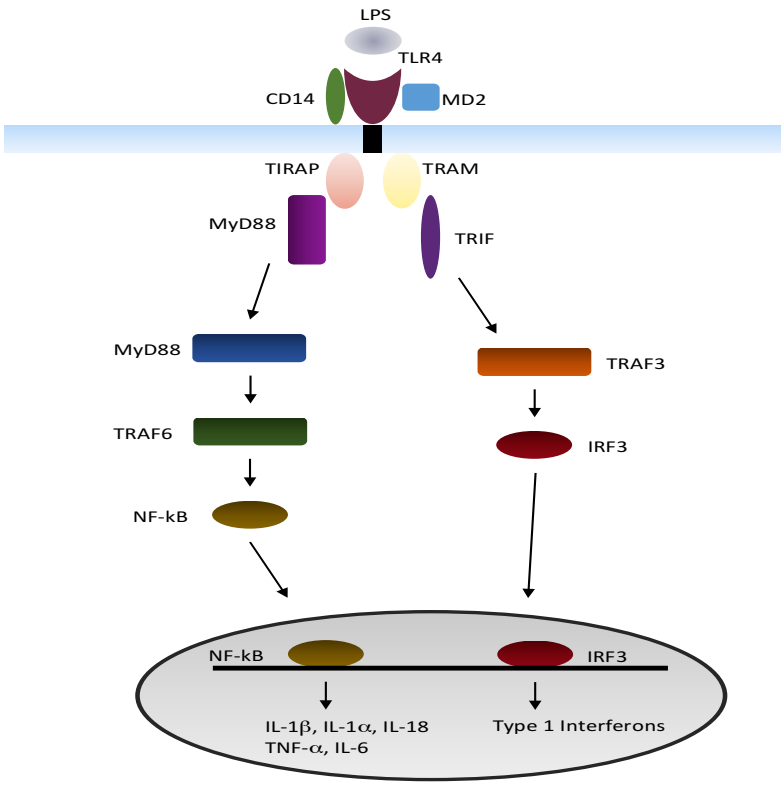


Figure 1. MyD88-dependent and MyD88-independent (TRIF) Signaling Pathway

Monophosphoryl Lipid A (MPL), a less toxic analogue of LPS, has been synthesized that retains the immunostimulatory properties of LPS and triggers a similar immune response (12, 15, 18, 19, 35, 36). The TLR4 agonist, MPL, is a chemically modified derivative of lipid A produced from *Salmonella Minnesota* R595 created by removal of its anomeric phosphate group and the acyl chain from the 3'-position of the disaccharide backbone (5, 6, 12). The adjuvant has ~0.1% of the inflammatory toxicity of LPS and drives the adaptive immune response by triggering a

cytokine cascade and activating antigen presenting cells via TLR4 (7, 8, 12, 15, 19, 31, 36). Thus, MPL is an essential compound for future vaccine adjuvants because of its ability generate a potent immune response without the inflammatory side affects associated with LPS (5, 7, 18, 19, 28). However, the mechanism underlying MPL's reduced toxicity remains unclear.

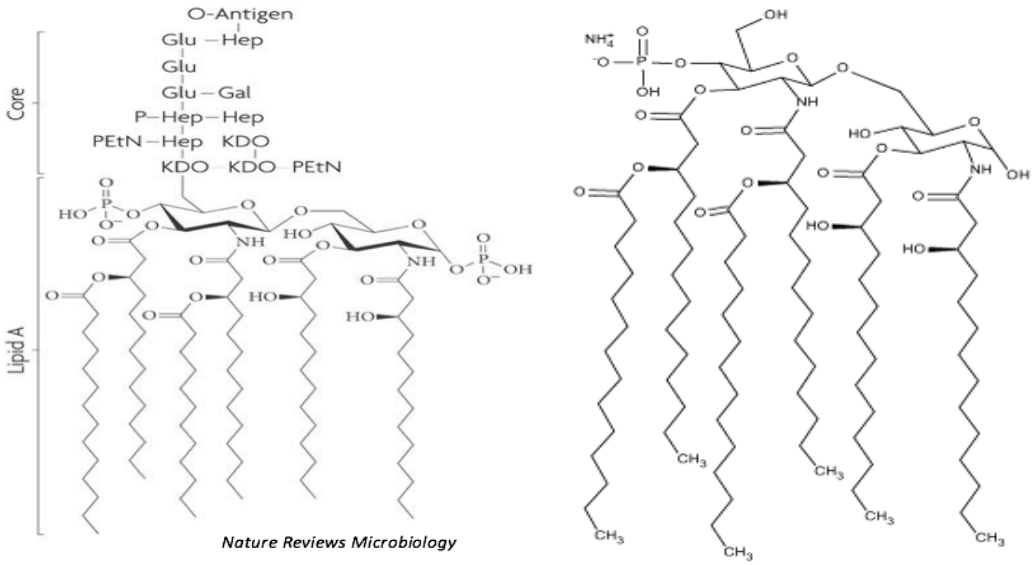


Figure 2. A comparison of the bacterial lipopolysaccharide (LPS) molecule (left) and synthetic form of monophosphoryl lipid A (MPL) or glucopyranosyl lipid A (GLA) molecule (right)

MPL Signaling is Primarily a TRIF Driven Pathway

Recent studies have proposed that the low toxicity associated with TLR4 adjuvant, MPL, is related to its bias towards the TRIF-dependent Pathway. These studies suggest that MPL's success as an adjuvant is attributed to its preference for the TLR4-TRIF pathway over the MyD88 signaling pathway (20, 38). The differential use of adaptor proteins leads to strong expression of gene products that are primarily TRIF-dependent while the MyD88 pathway is impaired and expression of proinflammatory cytokines reduced (29, 38). Results have implicated the activation of anti-inflammatory lipid phosphatase SHIP1 via the MyD88-dependent Pathway as a contributor to the decrease in cytokine expression (39). This is favorable in vaccinations

because of the molecule's ability to stimulate an immunostimulatory response without driving strong proinflammatory cytokine induction. Hence, the loss of MyD88 mediated cytokine induction implicates MPL as an effective immunostimulatory agent.

In the current study, we use a a synthetic form of MPL known as Glucopyranosyl Lipid A (GLA), to compare the impact of the modified derivative and LPS on the expression of critical cytokines (10). The activation of different signaling pathways in macrophages in response to LPS or GLA may be critical in explaining their difference in toxicity. To investigate the mechanisms underlying the reduced toxicity of GLA, we examined mRNA gene expression of key proinflammatory cytokines *in vivo* and *in vitro*. We determined the transcript levels of prototypical MyD88-dependent cytokine, TNF-α, and protypical TRIF-dependent cytokine, Type 1 Interferon IFNβ, to compare the inflammatory response following LPS or GLA stimulation in bone marrow derived macrophages (BMDMs). Understanding the response in BMDMs following TLR4 stimulation and a possible TRIF-dependent pathway bias would potentially reveal the value of the GLA adjuvant over the intact LPS molecule. The differential production of inflammatory cytokines would provide further insight into the effect of GLA in inducing a host immune response as well as increased understanding its role in future vaccine design.

Role of Host Signaling Molecule, Caspase 8, in LPS induced Endotoxic Shock

In addition, we attempt to determine the role of host signaling molecules downstream of TLR4 signaling in cytokine expression. Recent papers have expressed cell death mediating protease Caspase-8 (Casp8) to play a critical role in NF-κB signaling and gene expression following bacterial-induced macrophage cell death (2, 11, 44, 50). The signaling molecule has been implicated as crucial in mediating apoptosis in murine models and it has been established

that deletion of Casp8 leads to embryonic lethality in mice (2, 11). *Casp8*$^{-/-}$ mice have been observed to undergo necroptosis by Receptor-interacting protein-3 (RIP3) kinase but elimination of pro-necrotic kinase RIP3 produces a viable mouse (2, 11). Thus, our lab has developed a viable *Casp8*$^{-/-}$*RIP3*$^{-/-}$ double knockout mouse model that is resistant to apoptosis. In this study, we present data investigating the role of Casp8 in triggering TLR4 receptor mediated signaling cascades upon LPS and GLA exposure *in vivo*. Preliminary data from our lab show that *Casp8*$^{-/-}$ *RIP3*$^{-/-}$ (DKO) mice are resistant to lethality following high dose LPS injection while WT and *RIP3*$^{-/-}$ mice succumb to endotoxic shock. DKO mice. This implicates Casp8 as an essential component in TLR4 mediated signal transduction and the activation of crucial proinflammatory cytokines. Therefore, we sought to explore the role of Casp8 in TLR4 dependent TNF- α and IFNβ gene expression in the macrophages of LPS and GLA challenged mice. Identifying the role of critical cell death regulator Casp8 would provide insight into both LPS and GLA dependent TLR4 receptor stimulation and the inflammatory response that occurs in host defense. This would clarify the function of Casp8, specifically in Casp8-controlled pathways, and provide direction for the development of adjuvants in therapeutic settings. Our data suggests that Casp8 is indispensable following TLR4 mediated gene expression for both LPS and GLA at low dose challenge. Thus, the use of LPS derivative, GLA, as an adjuvant in any vaccine creation would require Casp8 because of its requirement in the transcription of critical proinflammatory cytokines TNF- α and IFNβ and the subsequent stimulation of the adaptive immune response.

OBJECTIVES

The following represent the objectives of this study:

1. To compare the expression of predominant cytokines produced by bone marrow-derived macrophages following LPS or GLA stimulation *in vitro*.

2. To determine role of host signaling molecules on cytokine expression in response to LPS or GLA injection *in vivo*.

MATERIALS AND METHODS

Reagents

Monophosphoryl Lipid A (synthetic) also known as Glucopyranosyl lipid adjuvant (GLA) was purchased from Enzo Life Sciences (Farmingdale, NY). Ultrapure Lipopolysaccharide from *Salmonella Minnesota* R595 was purchased from InvivoGen (San Diego, CA). The Anti-Mouse/Rat TNF-α antibody was purchased from eBioscience (San Diego, CA).

Preparation of Bone Marrow Derived Macrophages

For BMDM cultures, pooled bone marrow cells from flushed tibias and femurs were differentiated for 5 to 7 days in DMEM containing 20% fetal calf serum and 20% filtered L929-conditioned medium (to provide macrophage colony-stimulating factor).

RNA Isolation from Fresh Tissue and Cell Lines

Total RNA was isolated from cultured cells according to the manufacturer's instruction using the RNeasy Mini Kit from Qiagen (Valecia, CA). The quality and purity of RNA was assessed using the Thermo Scientific Nanodropp 2000 Spectrophotomer. RNA was reverse transcribed with Superscript First Strand Synthesis System for RT-PCR from Invivogen (San Diego, CA) using SuperScript II RT and Oligo(dT) to obtain first strand cDNA samples. Resulting cDNA was diluted 1:5 in nuclease free water and stored at -20°C until use for RT-PCR amplification.

Quantitative PCR Analysis

Gene expression levels were analyzed using real time, quantitative PCR. For mouse TNF-alpha amplification, the primers 5' – GAGGCACTCCCCCAAAAGAT - 3' and 5' – GAGGGAGGCCATTTGGGAAC - 3' were used. For IFN-β amplification, the primers 5' – CCTGGAGCAGCTGAATGGAA – 3'and 5' – CCACCCAGTGCTGGAGAAAT – 3' were used. Real-time PCR amplification was performed in a 20ul reaction mixture using SYBR Green Select Master Mix (Applied Biosystems) with the ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The thermal cycling conditions consisted of an initial denaturation step at 95° for 10 minutes, 45 cycles at 95° for 30 seconds, 60° for 30 seconds and 72° for 30 seconds. Measurements of gene expression were obtained in triplicate and the relative fold induction was determined using the Comparative Ct (ΔΔ CT) Method or as follows:

$$\Delta\Delta \text{ CT} = \Delta \text{ CT sample} - \Delta \text{ CT reference gene}$$

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

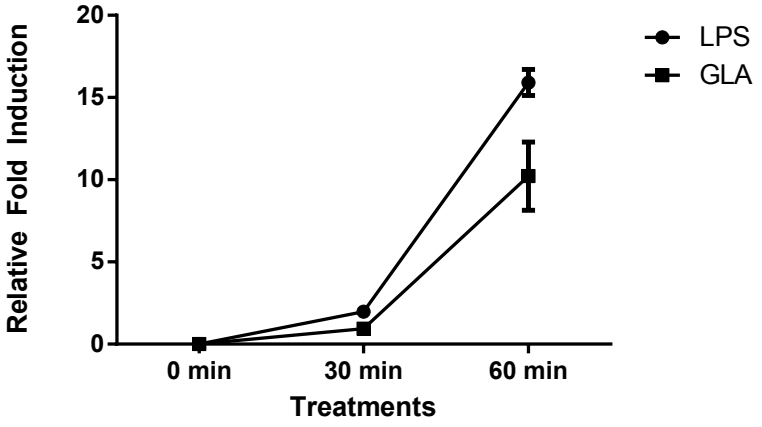
The reference gene GAPDH was used for normalization. Using this method, the fold changes of gene expression were determined.

RESULTS

TNF-α Gene Expression in Bone Marrow-Derived Macrophages after LPS or GLA stimulation

Macrophages respond to LPS or GLA treatments by releasing proinflammatory cytokines in a TLR4 dependent manner. To quantitatively evaluate the release of MyD88-dependent cytokine, TNF-α, by bone marrow-derived macrophages (BMDMs) after LPS or GLA treatment, we looked at mRNA transcript levels over a time-dependent and dose dependent response. BMDMs were stimulated with LPS or GLA at varying times and concentrations. Analysis of TNF-α gene expression revealed that GLA stimulated cells showed a reduction in mRNA production in comparison to LPS stimulated cells at each time point (Fig 3A). Quantification of gene expression also indicated decreased levels of TNF-α expression after GLA stimulation compared to LPS stimulation at each indicated dose (Fig 3B). Data is consistent with findings that show TNF-α cytokine production in GLA treated BMDMs is reduced compared to LPS treated BMDMs (12). Results suggest variation in cytokine expression levels after LPS or GLA treatment is due to differences in the signaling pathways after TLR4 stimulation.

A



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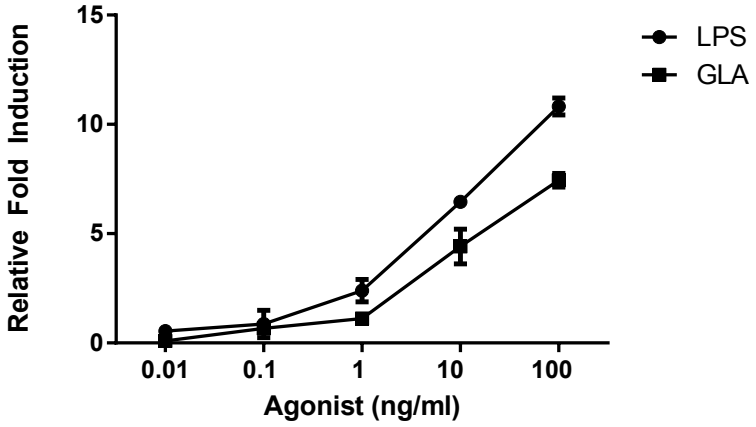

Figure 3. TNF-α Gene Expression in BMDMs after LPS or GLA stimulation. Wild-type BMDMs, plated in 6-well plates ($3x10^6$ cells/well) were treated with LPS (100 ng/ml) or GLA (100 ng/ml) for 0, 30, 60 min **(A)** or with 0.01 to 100 ng/ml of LPS or GLA for 60 minutes **(B).** Relative fold induction of TNF-α gene expression levels were measured using QT-PCR and Ct values were assessed. Data is from 2 independent experiments with triplicates in each experiment. Ct values were averaged for TNF-α and normalized to house-keeping gene GAPDH.

IFNβ Gene Expression in Bone Marrow-Derived Macrophages after LPS or GLA stimulation

To explore the effects of LPS or GLA stimulation in BMDMs on TRIF-dependent cytokines, we quantified IFNβ gene expression levels over a time-dependent response. Results reveal that GLA stimulated cells show similar levels of cytokine expression compared to LPS stimulated cells. The expression of IFNβ is slightly attenuated in GLA stimulated BMDMs compared to LPS stimulated BMDMs at 30 minutes. There is minimal difference in transcript levels between LPS and GLA stimulated BMDMs at 1 hour. Results show similar levels of IFNβ expression after LPS or GLA treatment over a time-dependent response. This data suggests that the MyD88-dependent pathway leads to decreased cytokine expression whereas the TRIF-dependent pathway is unaffected after GLA treatment.

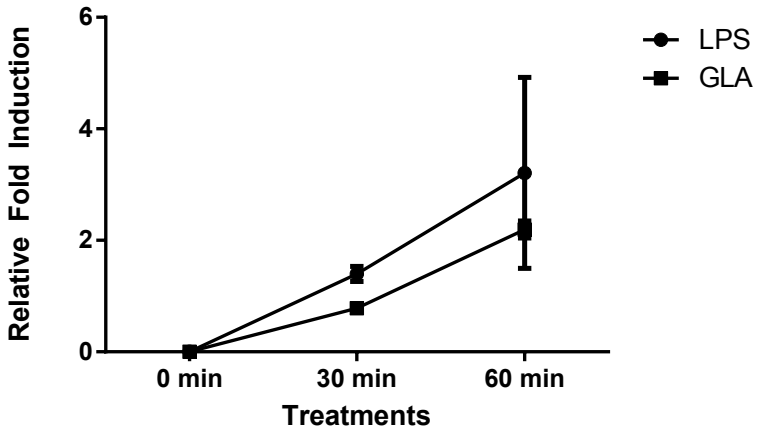

Figure 4. IFNβ gene expression in BMDMs after LPS or GLA stimulation. Wild-type bone marrow-derived macrophages plated in 6-well plates ($3x10^6$ cells/well) were treated with with LPS (100 ng/ml) or GLA (100 ng/ml) for 0, 30, 60 min. Relative fold induction of IFNβ gene expression levels were measured using QT-PCR and Ct values were assessed. Data is from 2 independent experiments with triplicates in each experiment. Ct values were averaged for IFNβ and normalized to house-keeping gene GAPDH.

Differential Expression of TNF-α by LPS and GLA is not explained by TLR4 saturation

A potential explanation for the different levels of proinflammatory cytokine expression by the TLR4 agonists is that more TLR4 receptors are being saturated by GLA than LPS. To test this hypothesis, we investigated the effects of adding LPS to pre GLA incubated BMDMs. We stimulated BMDMs with GLA for 1 hour before the addition of LPS at varying time points. Analysis of TNF-α gene expression levels reveals that the addition of LPS to GLA does not affect the differential levels of gene expression. This data suggests that TLR4s in BMDMs pretreated with GLA are not being stimulated more efficiently than LPS or GLA stimulated cells.

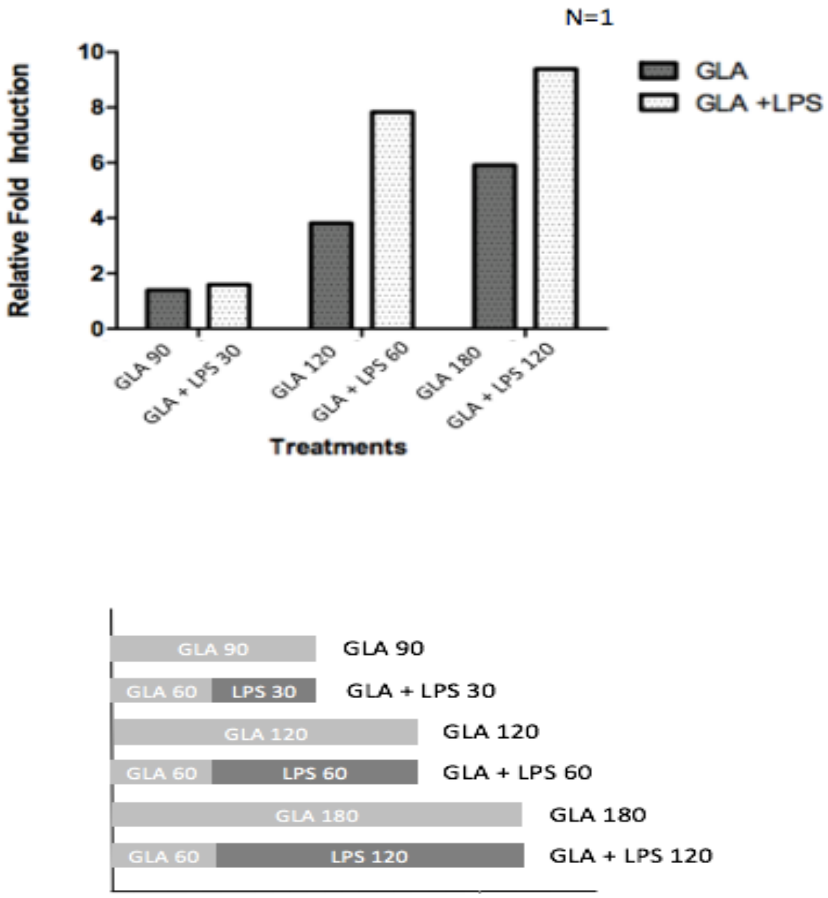

Figure 5. TNF-α Gene Expression in BMDMs pre-stimulated with GLA. WT BMDMs, plated in 6-well plates ($3x10^6$ cells/well) were stimulated with GLA at 50 ng/ml for 60 min. before LPS was added on at 50ng/ml for 30, 60, 120 min. Relative fold induction of TNF-α gene expression levels were measured using QT-PCR and Ct values were assessed. Data is from 1 independent experiment with triplicates in the experiment. Ct values were averaged for TNF-α and normalized to house-keeping gene GAPDH.

TLR4 Mediated Gene Expression by LPS or GLA Requires Caspase 8

The induction of proinflammatory cytokines and chemokines by myeloid cells such as macrophages is vital in the innate immune response. To extend our understanding of the role of host signaling molecule Casp8 in the immune response following LPS or GLA mediated TLR4 signaling, we investigated the contribution of Casp8 on the expression of MyD88-dependent cytokine TNF-α and TRIF-dependent cytokine IFNβ. Preliminary data from our lab has suggested that Casp8 is required in LPS induced endotoxic shock. Hence, we characterize the role of Casp8 by looking at the *in vivo* expression of two critical cytokines post LPS or GLA stimulation. We measured TNF-α and IFNβ gene expression of WT, *Casp8*$^{-/-}$*RIP3*$^{-/-}$ and *RIP3*$^{-/-}$ mice following a single intraperitoneal injection of either GLA (1 ug) or LPS (1 ug) along with relevant controls (17). We determined the gene expression levels of cytokines TNF-α and IFNβ from blood-derived macrophages 3 hours post injection using QT-PCR. LPS induced higher levels of TNF-α and IFNβ than GLA at 3 hours post injection in WT mice. A similar trend was observed in *RIP3*$^{-/-}$ mice in which LPS challenged mice had more elevated transcript levels of TNF-α and IFNβ than GLA challenged mice. In contrast, there was substantially less TNF-α and IFNβ genes expressed in *Casp8*$^{-/-}$*RIP3*$^{-/-}$ mice following LPS or GLA stimulation compared to both WT and *RIP3*$^{-/-}$ mice. Data suggests Casp8 is essential in TNF-α and IFNβ gene expression after LPS and GLA injection in mice.

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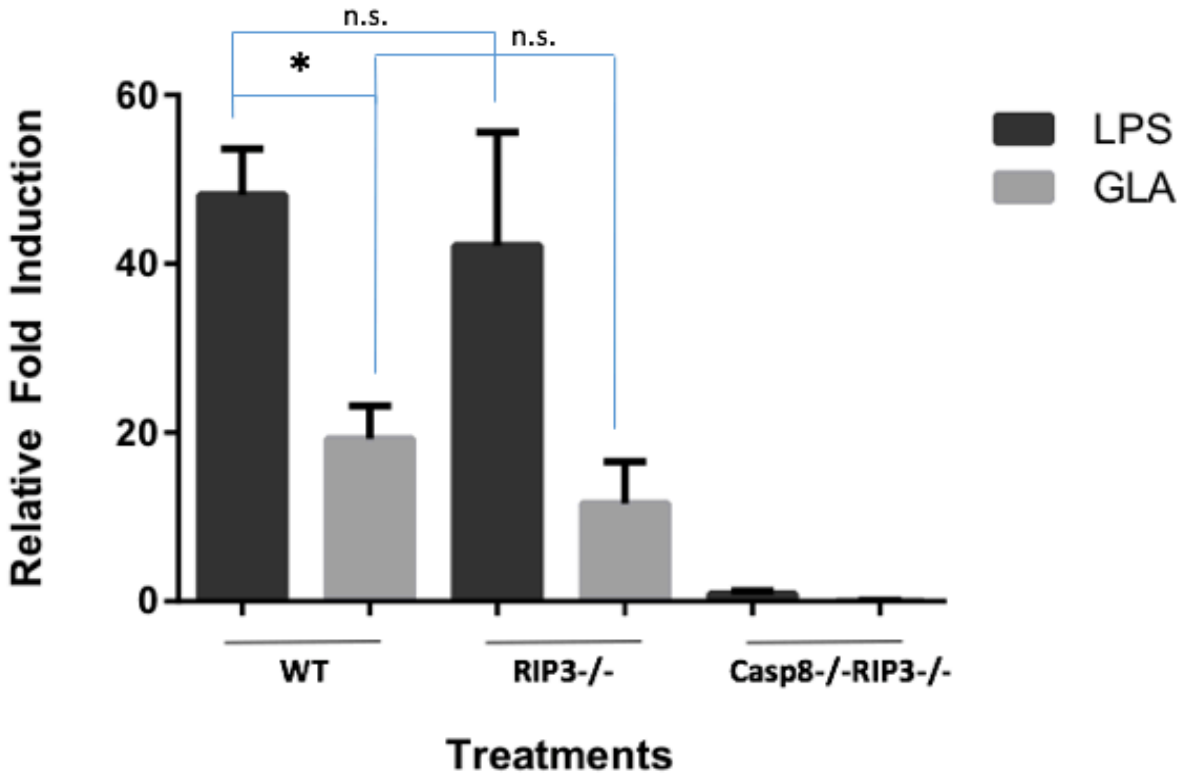
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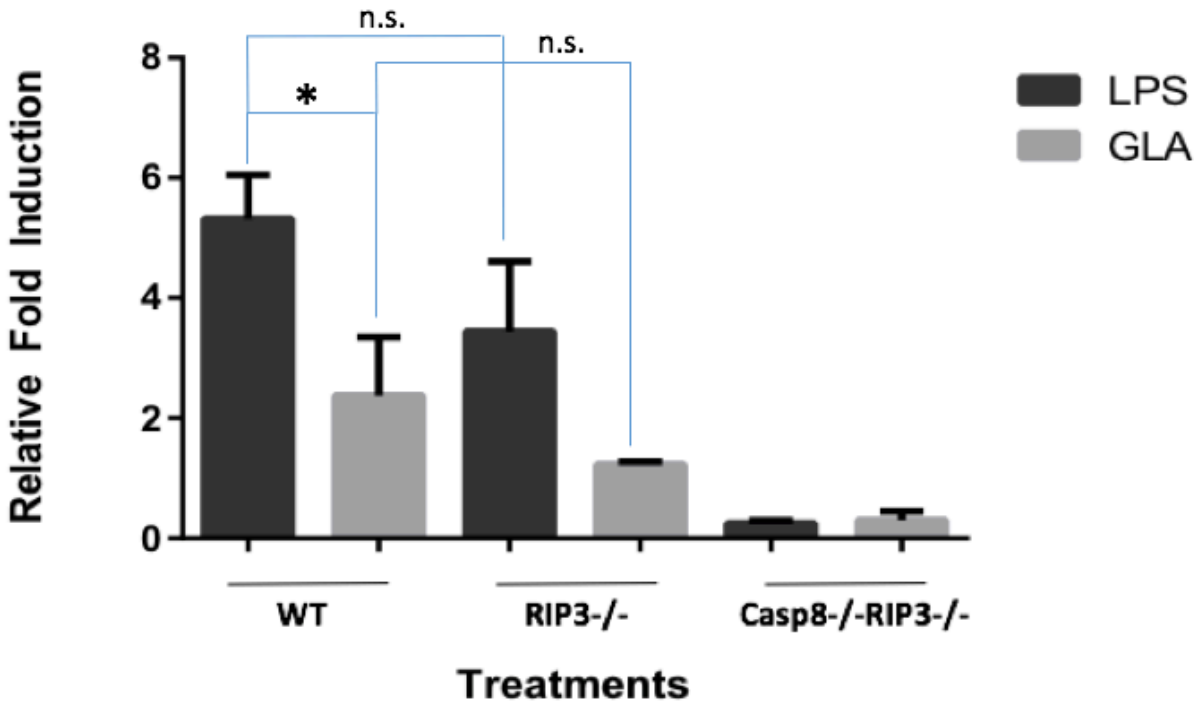
Casp8-/-RIP3-/-
Treatments

B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8
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Relative Fold Induction
LPS
GLA
WT
RIP3-/-
Casp8-/-RIP3-/-
Treatments

Figure 6. Gene expression in BMDMs in response to LPS or GLA stimulation *in vivo*. WT (n=4), *RIP3*$^{-/-}$ (n=3) and *Casp8*$^{-/-}$*RIP3*$^{-/-}$ (n=2) mice at 7-8 weeks old were injected intraperitoneally with PBS, GLA (1 ug) or LPS (1 ug). Mice were sacrificed 3 hours following injection and whole blood was harvested to obtain isolated RNA. TNF-α **(A)** and IFNβ **(B)** gene expression was quantified using QT-PCR and CT values were assessed. Data is shown in terms of relative fold induction from at least 2 independent experiments with triplicates in each experiment. Ct values were averaged for TNF-α and IFNβ and normalized to house-keeping gene GAPDH.

DISCUSSION

The ability of a host to mount an innate immune response primarily by APCs is critical following microbial infection and crucial to the organism's overall immune response. Understanding the inflammatory response as mediated by proinflammatory cytokines in response to adjuvants is critical in the continued development of effective vaccines and elimination of infectious disease. Septic shock resulting from LPS induced Gram-negative bacterial infection remains a leading cause of death worldwide. Lipid A is the biological portion of bacterial LPS responsible for its endotoxicity and immunostimulatory properties and has thus been evaluated for its ability to promote an immune response. It has been observed that the cell surface receptor TLR4 is essential for the induction of proinflammatory cytokines following LPS exposure (27). Hence, synthetic and natural TLR4 agonists have been recognized as potential targets in the development and design of alternative vaccine adjuvants. TLR4 agonist MPL, a less toxic derivative of LPS, has already been used in clinical trials and therapeutic vaccines (12). As a chemically modified form of MPL, the molecule produces an attenuated inflammatory response compared to LPS, while maintaining its adjuvanticity (27). Thus, comparing the downstream signaling pathways mediated by each molecule and their impact on the expression of proinflammatory cytokines will elucidate the properties and mechanisms responsible for MPL's reduced toxicity,

Stimulation of the TLR4 receptor on macrophages by LPS or the synthetic form of MPL, GLA, as used in this study, leads to the activation of two different signaling pathways: MyD88 signaling pathway and the TRIF signaling pathway (12, 25, 38, 41). These pathways facilitate the subsequent induction of critical cytokines and chemokines essential in promoting an immune response. The MyD88-dependent and TRIF-dependent signaling pathways are associated with the rapid activation of the innate immune response by proinflammatory cytokine induction and the

subsequent triggering of the adaptive immune response essential in vaccine creation (27). Recently, it has been reported that the low toxicity observed in MPL compared to LPS is associated with the TRIF drive signaling pathway and the reduction of MyD88-dependent cytokines expressed (38). In this study, our objective sought to provide insight into the impact of LPS or GLA stimulation on the TLR4 mediated gene expression of cytokines in mouse macrophages. We observed the mRNA transcript levels of TNF-α and IFNβ, released by the MyD88-dependent and TRIF-dependent pathway, respectively. Differences in cytokine protein levels have previously been observed following LPS or GLA treatment in mouse macrophages (12). Here, we identify that the difference is also seen transcriptionally. We report that LPS stimulated wild-type BMDMs generate enhanced TNF-α gene expression in comparison to GLA stimulated macrophages in a time-dependent and dose-dependent manner *in vitro*. These results support the conclusion that GLA stimulates the production of less MyD88-dependent cytokines than LPS does. Our data also reveals that similar transcript levels of IFNβ can be seen in both LPS and GLA treated macrophages. This result is consistent with the expectation that there should not be a difference in IFNβ gene expression when activated by the TLR4 mediated pathway. We were also interested in determining the contribution of TLR4 receptor stimulation to cytokine gene expression after LPS or GLA induced cell activation. Evidence shows that the expression of TNF-α following LPS exposure to pre-incubated GLA induced BMDMs was higher than GLA or LPS induced BMDMs. Therefore, it is unlikely that the differential levels of gene expression by LPS or GLA stimulation is caused by TLR4 receptor saturation. Further studies are needed to determine the precise cellular mechanism underlying GLA's reduced toxicity and TRIF-dependent signaling.

In addition, we identify the essential role of signaling molecule, Casp8, following TLR4 receptor stimulation in producing an inflammatory response by mRNA gene expression. Casp8 has

been implicated as a central contributor to LPS induced endotoxic shock in murine models. Preliminary data form our lab identify Casp8 as a critical mediator in TLR4 dependent shock after high dose LPS challenge *in vivo* by triggering apoptosis in intestine epithelial cells. It has been shown that Casp8 is required in the production of certain critical cytokines such as IL-1β downstream of TLR4 activation (45). Acknowledging that studies have identified GLA to stimulate a predominantly TRIF biased pathway with attenuated levels of MyD88-dependent cytokines, we looked at the role of Casp8 in producing proinflammatory cytokine TNF-α and Type 1 Interferon IFNβ *in vivo*. To determine the requirement of Casp8 following TLR4 activation by LPS or GLA, we injected wild-type, *RIP3*$^{-/-}$ and *Casp8*$^{-/-}$*RIP3*$^{-/-}$ mice with LPS or GLA at a low dose concentration of 1 ug, the dose at which vaccine adjuvants are commonly injected into the host. Results indicate that wild-type and *RIP3*$^{-/-}$ mice injected with GLA induced lower levels of TNF-α and IFNβ mRNA transcript levels compared to mice injected with LPS. Similar transcript levels were observed for both LPS and GLA challenged wild-type and *RIP3*$^{-/-}$ mice. Hence, RIP3 is dispensable for LPS and GLA induced gene expression. This data verifies the *in vitro* data in showing that TNF-α gene expression is decreased in GLA challenged wild-type mice. On the other hand, we did not expect to observe different transcript levels of TRIF-dependent cytokine, IFNβ, after LPS or GLA injection. The difference may be attributed to some level of cross-talk between the MyD88-dependent and TRIF-dependent pathways. However, *Casp8*$^{-/-}$*RIP3*$^{-/-}$ mice injected with either LPS or GLA led to significantly reduced and minimal levels of gene expression for both TNF-α and IFNβ. These findings suggest that Casp8 is required following TLR4 receptor mediated signaling and eventually producing an effective adaptive immune response. The reduction in gene expression by GLA stimulated mice suggests its role as a lower inflammatory agent compared to LPS and potential as a useful vaccine adjuvant in clinical settings. The lack of

gene expression by GLA following TLR4 receptor stimulation at a concentration level similar to that used in vaccine adjuvants indicates the importance of the protease in adjuvant design. GLA mediated stimulation of TLR4 may provide effective adjuvant activity by producing a potent immune response without toxicity that will facilitate in vaccine development.

Although the precise mechanism by which GLA or LPS acts to promote differential induction of inflammatory cytokines remains unclear, we identified that the reduced toxicity of GLA versus LPS occurs transcriptionally. Our study is also the first to identify that both LPS or GLA induced gene expression requires Casp8 *in vivo*. The results of this study have important implications for the future design of vaccine adjuvants using TLR4 agonists. Based on our studies, we demonstrate the potential of GLA as an adjuvant and provide evidence showing the advantages of the molecule in providing a protective host immune response without excessive inflammation.

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