Structure-Activity Relationship of Synthetic Toll-like Receptor 4 Agonists*

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Axel G. Stöver‡, Jean Da Silva Correia§, Jay T. Evans¶, Christopher W. Cluff**, Mark W. Elliott‡‡, Eric W. Jeffery‡, David A. Johnson§§, Michael J. Lacy¶, Jory R. Baldrige‡, Peter Probst‡, Richard J. Ulevitch§, David H. Persing††‡‡‡, and Robert M. Hershberg†††‡‡‡‡

From the ‡Corixa Corporation, Seattle, Washington 98104, ¶Corixa Corporation, Hamilton, Montana 59840, §Department of Immunology, The Scripps Research Institute, La Jolla, California 92037, and ¶¶The Infectious Disease Research Institute, Seattle, Washington 98104

Important questions remain regarding the impact of variations in the structure of the lipid A portion of lipopolysaccharide on activation of cells via the Toll-like receptor 4 complex. We have studied a series of synthetic lipid A mimetic compounds known as aminoalkyl glucosaminide phosphates in which the length of the secondary acyl chain has been systematically varied. Using transcriptional profiling of human monocytes and responses of Toll-like receptor 4 complex cell transfectants, we demonstrate a clear dependence of length on secondary acyl chain on Toll-like receptor 4 activation. Compounds with secondary acyl chains less than eight carbons in length have dramatically reduced activity, and substitutions of the left-sided secondary acyl chain had the most important effect on the Toll-like receptor 4 agonist activity of these molecules. The structure-function relationships of these compounds assessed via the induction of chemokines and cytokines following in vivo administration closely mirrored those seen with cell-based studies. This novel set of synthetic lipid A mimetics will be useful for Toll-like receptor 4-based investigations and may have clinical utility as stand-alone immunomodulators.

The ability of an organism to protect itself from microbial challenge requires the rapid recognition of "pathogen-associated molecular patterns," conserved structural motifs found throughout the microbial world. Toll-like receptors are a family of structurally related surface receptors that play an important role in transducing an inflammatory signal in response to pathogen-associated molecular pattern recognition. Toll was originally identified as a Drosophila gene essential for embryonic development and antifungal immunity (1). Ten mammalian orthologues of Toll-like receptors (TLRs)§§ have been identified (2–4). While all TLRs have similar structural features (e.g. extracellular leucine-rich repeats and cytoplasmic domains with homology to the interleukin-1 (IL-1) receptor), each TLR alone as a homodimer (e.g. TLR4, TLR5, TLR7, and TLR9) or in combination with another TLR as a heterodimer (e.g. TLR1, TLR2, and TLR6) is capable of recognizing distinct pathogen-associated molecular patterns due to unique structural motifs (5). Interestingly several natural and synthetic TLR ligands have been demonstrated to have significant clinical utility both as vaccine adjuvants and as stand-alone immunomodulatory agents (6–8).

One of the best studied interactions between a microbial pathogen-associated molecular pattern and a TLR is the recognition of lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria, by TLR4 (for a review, see Ref. 9). Beutler and colleagues (10) first identified TLR4 as the "LPS receptor" by positional cloning studies in the LPS hyposponderous mouse strains C3H/HeJ and C57BL/10ScCr. The critical role of TLR4 in the transduction of an inflammatory signal by LPS has been confirmed in studies using mice with a deletion of TLR4. In addition, two additional proteins have been identified that interact with TLR4 and are implicated in LPS recognition, MD-2 and CD14. MD-2 is a secreted molecule that physically interacts with TLR4 (11), and genetic data (12) suggest that this molecule is required for LPS signaling via TLR4. CD14 is a glycosylphosphatidylinositol-linked membrane protein (devoid of a cytoplasmic signaling domain) with LPS binding activity. Collectively TLR4, MD-2, and CD14 form a trimeric LPS receptor complex. The delineation of the precise molecular events associated with LPS binding and initiation of the signaling cascades via TLR4 remains an area of intense investigation.

Lipid A is the active component of the LPS molecule that stimulates the TLR4 receptor complex (13). Lipid A structures show considerable variability between bacterial species, and individual Gram-negative species can modulate the structure of the lipid A on their surface in the context of infection (14, 15). These structural differences, in particular the degree of acylation, can dramatically modify their proinflammatory capacity. In general, highly acylated (e.g. hexa-acylated lipid A from Escherichia coli or Salmonella species) tend to be potent inflammatory stimulators, while less acylated lipid A structures (e.g. lipidA,VA and lipid A from certain periodontal bacteria such as Porphyromonas gingivalis) tend to be less inflammatory or even display LPS antagonist activity (16). Because these studies have generally used natural biological preparations of LPS, cell expressed and secreted; MCP1, macrophage chemoattractant protein 1; MIP, monocyte inhibitory protein.
**Experimental Procedures**

**Chemical Synthesis of AGPs**—The compounds used in this study are shown in Fig. 1 and were synthesized as described previously (17). Aminoalkyl glucosaminide 4-phosphates or AGPs are a class of lipid A mimetics in which the reducing sugar of lipid A has been replaced with an N-acetylated aminoalkyl aglycon unit. The AGPs evaluated in this study (Fig. 1) contain an L-serine-based aglycon unit as well as three oligosaccharides that are attached to the lipid A core. For a detailed structure-function analysis of these AGPs and related molecules with defined modifications in the acyl chain length, using transcriptional profiling of human monocytes, responses of TLR4 receptor complex cell transfectants, and mouse and human primary macrophage-like cell responses, we were able to perform a detailed structure-function analysis of these AGPs and related molecules with defined modifications in the acyl chain length with changes in either of the sugar secondary acyl chains having a more dramatic effect on function of the molecules than changes in the aglycon secondary acyl chain. Furthermore, we highlight the potential utility of certain members of this novel class of compounds as stand-alone immunomodulators based on their TLR4 agonist activity.

**Structure-Activity of Synthetic TLR4 Agonists**

RNA Amplification, cDNA Synthesis, and Probe Labeling—Total RNA was amplified by the modified Eubrev ("antisense") RNA amplification protocol (19–21). All reagents were purchased from Invitrogen. After amplification, the concentration of antisense RNA was measured by spectrophotometry, and the quality was determined by gel electrophoresis. Amplified RNA (1.2 µg) was diluted in 16 µl of water and used as template for cDNA synthesis. All reagents were purchased from Invitrogen. Probes were labeled as described elsewhere (22).

Transfection of HeLa Cells and Reporter Assay—A HeLa cell transfection system was used to determine the roles of human TLR4, CD14, and MD-2 in the cellular response to AGPs. HeLa cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. HeLa cells were seeded at 1 x 10⁵ cells/well in a 12-well plate and transfected with 50 ng of IL-8 promoter-driven luciferase reporter and 10 ng of the indicated plasmids (23). The human CD14 cDNA used in this study was described previously (24).

Human TLR4 cDNA was a kind gift of Dr. C. Janeway. Eighteen hours after transfection, cells were stimulated for 6 h, lysed, and then analyzed for luciferase and β-galactosidase activities. A β-galactosidase construct was used for the normalization of enzyme activity (23). 293 cells were stably transfected with TLR2, then transiently transfected with TLR1 and TLR6, and exposed to heat-killed Staphylococcus aureus at a concentration of 1 µg/ml.

**Cytokine ELISA**—Monocyte-derived macrophages were cultivated as described elsewhere (18). After 5 days of culture, the medium was removed and replaced with fresh medium to which AGPs were added at the concentrations indicated. After 24 h, the supernatants were collected and stored at −20 °C until the day of analysis. Supernatants were analyzed for TNF-α by ELISA assay using an anti-human TNF-α antibody and a biotinylated anti-human TNF-α antibody (BD Biosciences).

**Close Selection and PCR**—Numerous human genes of interest were selected from the literature, and their cDNAs and expressed sequence tags were identified in GenBank™. This list of genes of interest included cytokines and cytokine receptors, chemokines and chemokine receptors, TLRs and TLR-associated proteins, adhesion molecules, nuclear factor-κB signaling in macrophages, a number of NFκB-controlled genes, apoptosis-related genes, and numerous miscellaneous genes of interest. Most of the cDNAs and expressed sequence tags used in this study were purchased from Research Genetics Invitrogen. A small number of clones were extracted from our in-house clone collections. E. coli clones were streaked on Luria-Bertani (LB) agar plates supplemented with an appropriate antibiotic, and single colonies served as the inoculum for the high throughput cultivation on 96-well plates (100 µl/well). After 24 h of cultivation, 1 µl of this cell suspension was collected and used as template to amplify plasmid inserts by PCR. PCR products were then screened on 1% agarose gels, purified using 96-well multiscreen PCR plates (Millipore, Bedford, MA), and sequenced. After obtaining a number of NFκB-controlled genes, apoptosis-related genes, and numerous miscellaneous genes of interest, suitable PCR products were transferred to 384-well plates and stored at −80 °C prior to printing.

**Printing of Arrays**—Approximately 1200 PCR products and controls were spotted onto 42 CMT-GAPS2 coated slides in triplicate (Corning) using an Affymetrix 417 arrayer (Affymetrix, Santa Clara, CA). Humidity control was maintained in the range of 45–52% using an ultrasonic humidifier (Model 696, Sunbeam, Boca Raton, FL). Following printing, the arrayed slides were UV cross-linked at 90 mJ overnight (Stratalinker, Stratagene, La Jolla, CA) and baked for 2 h at 80 °C until the day of hybridization. The slides were prehybridized for a minimum of 45 min in a buffer consisting of 5 x SSC, 1% bovine serum albumin, 25% formamide, and 0.1% SDS. Overall array quality was confirmed by hybridization to a Cy3 end-labeled, M13-reverse oligonucleotide (Qiagen Operon, Alameda, CA).

**Probe Labeling and Hybridization**—Fluorescently labeled probes were generated from 1.5 µg of antisense RNA amplified as described above. The Cy3- (treated) or Cy5 (untreated)-labeled probes were generated using the amino-allyl method described elsewhere (25) and included in the microarray resources section of The Institute of Genomic Research (TIGR) Web site. To determine the sensitivity of the microarray hybridization, the target RNA and the control RNA (Spot Report-3, Stratagene) were spiked into each probe sample prior to cDNA synthesis. The corresponding Arabidopsis CDNAs were arrayed on each slide with a variety of human housekeeping control genes (β-actin, α-tubulin, phospholipase A₂, transferrin, ribosio-
mal protein a9, highly basic protein, and major histocompatibility complex class I) in each of the four quadrants of the array. Probes were checked for successful labeling by spectrophotometry using a 2- or 5-μL cuvette and a GeneSciences 3 unit (MiraiBio Inc., Alameda, CA). Hybridizations and posthybridization washes were carried out as described by the slide manufacturer (Corning) for MeSO arrays using a formamide hybridization buffer. Hydrophobic coverslips (Hybrislip, Grace Biolabs, Bend, OR) were used in place of glass coverslips. Two individual hybridizations were performed for each sample with similar results, and results of one hybridization are shown.

Microarray Data Acquisition and Analysis—Following washing of arrays, slides were scanned using an Affymetrix 418 microarray scanner and saved as 16-bit TIFF files (Affymetrix). The Cy3 and Cy5 images for each array were overlaid, gridded, and quantified using Imagene software (Biodiscovery, Marina del Rey, CA). Hybridization data quality was evaluated using the mean signal to noise ratios for all cDNA spots, the mean signal to noise ratios for control spots, treated to untreated ratios for control, and spike-in spots and spot morphology. Normalization and further data analysis was performed using GeneSpring (Silicon Genetics, Redwood City, CA). The hybridization intensity of each spot was normalized to the median intensity of all non-control spots on the array. One-sample Student’s t test values (Table 1) were calculated by GeneSpring to determine whether the mean normalized expression levels were statistically different from 1.0 (treated versus untreated).

In Vivo Characterization of AGPs—To test whether AGPs stimulate the innate immune system in vivo, each of the AGPs and LPS were dissolved in triethylammonium salts and injected at various concentrations into BALB/c mice (Jackson Laboratories, Bar Harbor, ME) via the intravenous route (tail vein, five mice/group). Blood was collected 2 h following administration of the compounds, and the concentrations of serum cytokines (TNF-α, IL-1β, IL-6, and IL-10) and chemokines (RANTES, MCP1, MIP1α, and KC) were determined using a custom Lincoplex cytokine array (Linco Research, St. Charles, MO) and a Luminex 100 IS system (Luminex, Austin, TX). The results presented are the average of five mice/group with the error bars based on S.E. values.

RESULTS

Chemical Structure of the Synthetic Lipid A Mimetics, AGPs—We previously described the synthesis of the AGP class of compounds, which are composed of a monosaccharide unit glycosidically linked to an N-acylated aminooalkyl aglycon unit (17). Chemical synthesis readily permits variations in the fatty acid residues and the chemical nature and charge of the aglycon unit. In this study, we focused on changes in acyl chain length within a family of AGP molecules possessing an L-serine-based aglycon unit, the so-called "L-seryl" family. As shown in Fig. 1, every member of this family has 14 carbon atoms in the "primary" fatty acid, which is the β-hydroxy fatty acid attached directly to the AGP backbone. β-Hydroxyxymyristic acid is the most common of the primary fatty acids present in lipid A. With respect to the normal or secondary fatty acid chain length, we have synthesized compounds with 6-, 8-, 10-, 12-, and 14-carbon acyl chains (Fig. 1A). In addition, we prepared a series of hybrid molecules based on the structure of CRX-527 (10-10-10) in which one or two of the 10-carbon acyl chains of CRX-527 (10-10-10) have been replaced with a six-carbon acyl chain (Fig. 1B). Collectively these compounds were used in a variety of cell-based and an in vivo model to determine structure-activity relationships related to AGP acyl chain length and stimulation via TLR4.

Cytokine Production by Human Monocytes Is Dependent on AGP Acyl Chain Length and Position—While TLR4 is expressed on a variety of hematopoietic and non-hematopoietic cells, monocytes and macrophages have been frequently studied as physiologically relevant LPS-sensitive cells (26, 27). The hallmark of monocyte and macrophage activation through Toll-like receptors is the activation of NFκB and the subsequent release of TNF-α and other cytokines and chemokines (28). To determine the activity of our synthetic lipid A mimetic, AGPs, we exposed human monocyte-derived macrophages (21) from three donors to various concentrations of these compounds and measured released TNF-α in the supernatant by ELISA. Fig. 2 shows the ELISA results for TNF-α presented as the average response from three donors. As shown in Fig. 2A, the amount of TNF-α release in response to the AGPs varied as a function of the secondary acyl chain (SAC) length. Specifically treatment of cells with AGPs carrying SACs 10 carbons or greater in length (CRX-527 (10-10-10), CRX-560 (12-12-12), and CRX-512 (14-14-14)) resulted in the production of TNF-α (Fig. 2A). In marked contrast, the compounds with SACs less than 10 carbons (CRX-526 (6-6-6) and CRX-555 (8-8-8)) did not elicit the production of TNF-α by the same cells (Fig. 2A) alone or with co-incubation of the cells with interferon γ (data not shown). Co-administration of interferon γ to the cells in addition to the respective AGPs augmented the induced levels of TNF-α observed but did not alter the relationship between acyl chain length and activity (data not shown). We consistently found that the compound with the 10-carbon SAC (CRX-527 (10-10-10)) was the most potent tested, maximally active at a concentration of less than or equal to 1 ng/ml. Compounds with SAC lengths of 12 and 14 carbon atoms were considerably less potent than CRX-527 (10-10-10) and showed a more clear dose-response relationship in the ng/ml to μg/ml concentration range (Fig. 2A). The same potency relationship was seen when these compounds were tested for the production of TNF-α using the U937 and THP-1 monocyte/macrophage cell lines (data not shown).

These data highlighted a clear dependence on SAC length with regard to the stimulatory capacity of synthetic lipid A mimetics. Given the fact that CRX-526 (6-6-6) showed no activity in these assays and CRX-527 (10-10-10) was maximally active at less than or equal to 1 ng/ml, we evaluated compounds in which six-carbon acyl chains (hexanoic acid) were inserted into each position in CRX-527 (10-10-10). These compounds, shown in Fig. 1B, allowed us to further define the structural features of the lipid A molecule required for macrophage stimulation. As shown in Fig. 2B, substitution of one or two hexanoic acid chains into CRX-527 (10-10-10) dramatically reduced the activity of the molecule with regard to TNF-α production by human macrophages.

Analysis of Transcriptional Activation of Macrophages by AGPs—While our data clearly demonstrate the ability of AGPs to induce TNF-α production in human macrophages, we were interested in evaluating the structure-activity relationship associated with the more complex transcriptional response following stimulation with AGP compounds.

To this end, we developed a custom microarray containing ~300 inflammatory target genes with each target arrayed in triplicate to allow quality control and statistical analysis on each microarray slide. Total RNA derived from human macrophages stimulated with individual AGPs for 6 h was used as a template to generate Cy3- or Cy5-labeled cDNA used for hybridization to the microarray. In preliminary experiments, the 6-h time point was observed to result in the best signal to noise ratio of the majority of genes tested (data not shown). Identical cells stimulated with LPS from E. coli 0127:B8 served as a positive control. As shown in Fig. 3A, LPS stimulated the expression of 64 array elements more than 2-fold (t test p value for each element <0.05) compared with the complete lack of induced gene expression seen in the negative control (triethylammonium salts vehicle).

The analysis of the AGP-stimulated macrophage gene expression on this microarray confirmed the strict dependence on SAC length seen with the induction of TNF-α release by these cells. Specifically CRX-526 (6-6-6) did not induce any genes on
the array. While CRX-555 (8-8-8) did not induce TNF-α release in the macrophages when assayed by ELISA (Fig. 2A), we observed the induction of eight genes on the microarray following stimulation with this compound in the same cells. A dramatic increase in the transcriptional activation was seen with 68 genes induced by CRX-527 (10-10-10) and 59 and 50 genes
induced by CRX-560 (12-12-12) and CRX-512 (14-14-14), respectively.

We also evaluated the transcriptional responses elicited by the substituted AGP compounds using the same microarray. As shown in Fig. 3A, the substitution of two decanoic acid chains with hexanoic acid chains dramatically resulted in a loss of stimulatory activity compared with CRX-527 (10-10-10). Interestingly, given the increased sensitivity of the microarray analysis compared with the TNF-α ELISA data, several of the compounds with substitutions of a single hexanoic acid onto the 10-10-10 backbone induced modest transcriptional responses (Fig. 3A). Specifically this partial response was seen with CRX-565 (10-6-10) and CRX-569 (10-10-6), compounds in which the left-sided decanoic acid was preserved. In marked contrast, CRX-566 (6-10-10) was essentially non-stimulatory, mirroring the lack of responsiveness seen with the doubly substituted compounds or CRX-526 (6-6-6).

The list of 68 genes affected by CRX-527 (10-10-10) is presented in Table I. Because stimulation with CRX-527 (10-10-10) resulted in the most significant transcriptional response of all of the AGPs tested, we focused our comparative analysis on the genes that were either up- or down-regulated by CRX-527 (10-10-10) with a t test p value of less than or equal to 0.05. Organization of the expression data by performing hierarchical clustering (29) into a gene tree was performed and is shown in Fig. 3B. The profiles of gene induction by LPS and CRX-527 (10-10-10) are very similar with LPS being a modestly stronger stimulant. Two exceptions to this rule are the small inducible cytokine SCYB10 and the monokine induced by interferon γ.
Table I

Expression data (-fold scale) of genes affected in monocyte-derived macrophages are shown. Genes were selected based on stimulation with CRX-527 (10-10-10) (expression of \( \pm 2 \) or \( \pm 0.5 \)-fold with a \( t \) test \( p \) value \( \leq 0.05 \)) and are compared with CRX-560 (12-12-12), CRX-560 (14-14-14), and LPS. MIG, monokine induced by interferon γ; ICAM1, intercellular adhesion molecule 1; SOCS-1, suppressor of cytokine signaling-1; TRAF1, tumor necrosis factor receptor-associated protein 1; IRF1, interferon regulatory factor 1; TNFR, TNF receptor; TGF, transforming growth factor; STAT, signal transducers and activators of transcription; COX-2, cyclooxygenase-2; TAP1, transporter associated with antigen processing 1; HSP, heat shock protein; CSF-1, colony-stimulating factor-1.

| Gene                | Treatment              | CRX-527 (10-10-10) | CRX-560 (12-12-12) | CRX-512 (14-14-14) | LPS   |
|---------------------|------------------------|---------------------|---------------------|---------------------|-------|
| **Chemokines**      |                        |                     |                     |                     |       |
| SCYB10              | 46.4                   | 6.6                 | 3.1                 | 23.4                |       |
| SCYA8               | 33.4                   | 25.3                | 19.6                | 41.6                |       |
| SCYA4               | 23.6                   | 16.6                | 14.5                | 23.2                |       |
| Exodus-1            | 22.0                   | 31.9                | 33.6                | 42.2                |       |
| SCYB11              | 20.8                   | 3.9                 | 1.3                 | 10.7                |       |
| RANTES              | 16.3                   | 14.7                | 6.0                 | 24.5                |       |
| MIP3α               | 15.3                   | 11.6                | 19.0                | 34.9                |       |
| SCYA1               | 13.0                   | 17.0                | 9.6                 | 20.4                |       |
| MIG                 | 11.1                   | 2.2                 | 1.3                 | 5.2                 |       |
| MIP3β               | 7.9                    | 7.4                 | 2.4                 | 11.0                |       |
| SCYB13              | 7.7                    | 7.0                 | 1.3                 | 10.4                |       |
| IL-8                | 3.6                    | 6.4                 | 9.2                 | 7.0                 |       |
| MIP2α               | 3.5                    | 7.2                 | 7.4                 | 8.3                 |       |
| SCYA13              | 2.4                    | 2.1                 | 1.9                 | 2.8                 |       |
| CCR5                | 2.2                    | 1.6                 | 1.4                 | 1.5                 |       |
| **Cytokines**       |                        |                     |                     |                     |       |
| IL-6                | 27.0                   | 17.4                | 25.5                | 61.1                |       |
| IL-2 receptor-α     | 19.4                   | 17.2                | 11.1                | 27.5                |       |
| SOCS-1              | 19.3                   | 5.3                 | 4.9                 | 6.9                 |       |
| TNF-α               | 10.8                   | 5.6                 | 7.6                 | 9.5                 |       |
| TRAF1               | 8.1                    | 4.5                 | 4.6                 | 6.8                 |       |
| TNFAIP3             | 8.1                    | 3.8                 | 3.0                 | 5.5                 |       |
| TNFRSF5             | 5.5                    | 4.0                 | 3.0                 | 4.8                 |       |
| IL-1RA              | 3.5                    | 2.2                 | 1.9                 | 3.0                 |       |
| IRF1                | 3.3                    | 2.8                 | 1.9                 | 3.0                 |       |
| IL-15               | 2.9                    | 2.0                 | 1.7                 | 2.9                 |       |
| TNFRSF1B            | 2.8                    | 2.7                 | 1.9                 | 2.8                 |       |
| **Surface receptors**|                       |                     |                     |                     |       |
| CD38                | 20.0                   | 12.3                | 3.8                 | 20.3                |       |
| CD80                | 4.2                    | 2.6                 | 2.7                 | 5.7                 |       |
| CD44                | 4.1                    | 3.5                 | 2.6                 | 3.6                 |       |
| ICAM1               | 3.5                    | 2.9                 | 2.9                 | 3.1                 |       |
| Prostaglandin F receptor-α | 2.9 | 2.6 | 2.7 | 3.5 |       |
| Prostaglandin E receptor-α | 2.1 | 1.9 | 2.2 | 1.4 |       |
| TLR5                | 0.3                    | 0.3                 | 0.5                 | 0.3                 |       |
| TGF-β receptor 2    | 0.3                    | 0.4                 | 0.5                 | 0.3                 |       |
| Interferon γ receptor 1 | 0.3 | 0.0 | 0.6 | 0.4 |       |
| CD14                | 0.3                    | 0.8                 | 1.0                 | 0.5                 |       |
| TGF-β receptor 1    | 0.3                    | 0.4                 | 0.5                 | 0.3                 |       |
| **Signaling**       |                        |                     |                     |                     |       |
| STAT4               | 7.9                    | 6.5                 | 4.5                 | 9.4                 |       |
| STAT2               | 6.3                    | 3.1                 | 1.6                 | 4.4                 |       |
| MyD88               | 3.3                    | 2.1                 | 1.6                 | 2.3                 |       |
| Lyn tyrosine kinase | 2.9                    | 2.6                 | 2.3                 | 2.6                 |       |
| MAP3K5              | 1.9                    | 1.9                 | 1.7                 | 1.8                 |       |
| **Transcription**   |                        |                     |                     |                     |       |
| NFκB1A              | 5.0                    | 3.7                 | 3.2                 | 4.9                 |       |
| NFκB1 (p105)        | 4.6                    | 2.4                 | 2.7                 | 2.5                 |       |
| ETS2                | 2.6                    | 3.5                 | 2.6                 | 3.6                 |       |
| **Miscellaneous**   |                        |                     |                     |                     |       |
| COX-2               | 14.9                   | 25.6                | 20.5                | 42.1                |       |
| Pentraxin-3         | 13.4                   | 11.8                | 16.1                | 20.8                |       |
| TAP1                | 5.5                    | 4.6                 | 1.9                 | 4.3                 |       |
| Proteasome subunit, β-type, 9 | 4.0 | 3.3 | 1.3 | 4.3 |       |
| IEE2                | 3.7                    | 4.6                 | 4.1                 | 3.7                 |       |
| Caspase 7           | 3.7                    | 2.2                 | 1.5                 | 2.7                 |       |
| Proteasome subunit, α-type, 2 | 3.3 | 2.0 | 1.3 | 2.2 |       |
| FOSIL2              | 3.2                    | 0.0                 | 2.5                 | 3.2                 |       |
| LAK-1               | 3.2                    | 1.4                 | 1.2                 | 1.9                 |       |
| CARD15              | 3.0                    | 2.2                 | 1.2                 | 2.7                 |       |
| Caspase 4           | 3.0                    | 2.3                 | 2.2                 | 3.2                 |       |
| Caspase 1           | 2.3                    | 2.0                 | 1.7                 | 1.9                 |       |
| β-Defensin-1        | 2.3                    | 1.2                 | 1.1                 | 2.8                 |       |
| HSP 70-kDa 1A       | 2.2                    | 1.5                 | 1.1                 | 1.6                 |       |
| TGFA                | 2.1                    | 1.7                 | 1.4                 | 2.3                 |       |
| Proteasome subunit, α-type 3 | 2.0 | 1.4 | 1.3 | 1.4 |       |
| WASP1               | 0.5                    | 0.7                 | 0.5                 | 0.4                 |       |
| Rho GDP dissociation inhibitor (GDI) β | 0.4 | 0.5 | 0.6 | 0.4 |       |
| Sprouty 2           | 0.4                    | 0.5                 | 0.7                 | 0.3                 |       |
| CSF-1               | 0.3                    | 0.7                 | 0.4                 | 0.4                 |       |
| Leukotriene-A4 hydrolase | 0.3 | 0.3 | 0.4 | 0.2 |       |
| Arachidonate 5-lipoxygenase | 0.3 | 0.4 | 0.4 | 0.3 |       |
| Plasminogen activator, urokinase-type | 0.1 | 0.4 | 0.4 | 0.2 |       |
(MIG; CXCL9); the transcripts of these genes are more than twice as abundant when stimulated by CRX-527 (10-10-10) when compared with LPS (46.4-fold versus 23.4-fold and 11.1-fold versus 5.2-fold, respectively). While some genes may not be present in the LPS-induced sample due to filtering of the data set using a strict cutoff (t test p value of 0.05), in general, we were not able to detect any genes specifically induced by any of the stimulatory AGPs tested alone (CRX-527 (10-10-10), CRX-560 (12-12-12), CRX-512 (14-14-14), CRX-565 (10-6-10), and CRX-569 (10-10-6)) that were not induced by LPS. The transcriptional profiles of CRX-527 (10-10-10) and CRX-569 (10-10-6) are highly similar. Likewise CRX-560 (12-12-12) and CRX-512 (14-14-14) clustered together with LPS, indicating that these two AGPs and LPS similarly affect the expression of the 149 array elements analyzed. CRX-555 (8-8-8) and CRX-566 (6-10-6) also cluster together, suggesting strong similarities between the effects of these two AGPs. Most strikingly, the non-stimulatory AGPs CRX-526 (6-6-6), CRX-567 (6-6-10), and CRX-568 (6-10-6) cluster together with the vehicle control triethylammonium salts.

Analysis of AGPs Using Transfectants Expressing the TLR4 Receptor Complex—TLR4 is expressed on the surface as part of a trimolecular complex with both MD-2 and CD14 (23). MD-2 has been shown to physically associate with nascent TLR4 in the endoplasmic reticulum but is also secreted and interacts with the extracellular domain of TLR4 expressed on the cell surface (30). Based on biochemical and genetic data, MD-2 is required for LPS-induced stimulation via TLR4. CD14 is a glycosylphosphatidylinositol-linked protein to which LPS has been shown to be transferred via lipopolysaccharide-binding protein circulating in the serum (31). We have previously demonstrated that HeLa cells directed to express TLR4, MD-2, and CD14 following transient transfection respond to LPS (23). We have used a luciferase reporter plasmid containing a portion of the IL-8 promoter, known to be readily induced by LPS via the activation of NFkB, as readout in this assay.

HeLa cells were transiently transfected with TLR4 alone, or in combination with MD-2 and/or CD14, and stimulated with varying concentrations of AGPs. As shown in Fig. 4A, HeLa cells transfected with TLR4 and MD2 gained responsiveness to LPS and CRX-527 (10-10-10); co-expression of CD14 potentiated the response (Fig. 4, B and E). Cell supernatants of HeLa cells were also examined for the production of IL-8 by ELISA, and a similar pattern of response was observed (data not
shown). In addition, HeLa cells transfected with *Drosophila* Toll (with or without MD-2) or with TLR2 alone were not stimulated by LPS or the AGPs (data not shown). The ability of the AGPs to stimulate the transfectants was strictly dependent on the length of the SAC with results closely mirroring those seen with the human macrophages. Co-expression of CD14 in the transfectants was not required for stimulation but did augment responses to the stimulatory AGPs, particularly at lower concentrations and with the compounds with suboptimal SAC lengths (*e.g.* 8- and 14-carbon, Fig. 4B). Similar to the human macrophage cytokine and microarray results, we did not see any TLR4-mediated activation by CRX-526 (6-6-6), which contains hexanoic acid residues in all three of the secondary positions. These data demonstrate the TLR4 dependence and specificity of stimulation with the AGP compounds.

We similarly evaluated a set of partially substituted AGP compounds in this TLR4 transfectant system. As seen in the microarray analysis of stimulated human monocytes (Fig. 3), we found that compounds with a single six-carbon acyl chain in the middle or right position (CRX-565 (10-6-10) and CRX-569 (10-10-6)) in the context of the 10-carbon SAC structure had significant activity in the TLR4/MD-2 transfectants (Fig. 4C). The compound with a substitution of a six-carbon acyl chain, the left-sided SAC (CRX-566 (6-10-10), which showed minimal activation via microarray) was similarly unable to stimulate the TLR4/MD-2 transfectants. Interestingly co-expression of CD14 with TLR4 and MD-2 conferred slight responsiveness to CRX-566 (6-10-10) and augmented the responses to CRX-565 (10-6-10) and CRX-569 (10-10-6) (Fig. 4D). Consistent with the data from the microarray experiment, the compounds in which two hexanoic acids were substituted for decanoic acids had minimal to no stimulatory capacity in the transfectants (Fig. 4D). These data further highlight the relative importance of the left-sided acyl chain length in lipid A structures in stimulation via the TLR4 complex.

To determine whether TLR1, TLR2, or TLR6 could respond to the AGP, experiments were carried out with 293 cells expressing TLR2 in the presence or absence of TLR1 and TLR6. CRX-527 (10-10-10) did not activate cells through TLR2, TLR1, or TLR6 alone or in combination (Fig. 4E). In these latter experiments, heat-killed *S. aureus*, a potent stimulus of TLR2, was used as positive control. In addition, HeLa cells transfected with *Drosophila* Toll (with or without MD-2) were not stimulated by LPS or the AGPs (data not shown).

**Analysis of AGP Structure-Function Relationships following in Vivo Administration**—The analysis of the response of human macrophages and TLR4 complex transfectants to the AGP compounds has demonstrated the potent activity of these compounds and a substantial dependence on the activity with SAC length. To extend these observations to an *in vivo* model, we determined the effect of intravenous administration of these AGP compounds on cytokine and chemokine production in BALB/c mice. Mice were injected with five different doses of AGPs, and serum was analyzed after 2 h for levels of RANTES, MCP-1, MIP1α, KC (a mouse IL-8 homologue), IL-1β, TNF-α, IL-10, and IL-6. Levels of RANTES, MCP-1, MIP1α, and KC following AGP administration are presented in Fig. 5 and were representative of the patterns observed with all of the cytokines and chemokines tested. In all cases, there was a strict dependence on SAC length that closely mirrored that seen in human macrophages and the TLR4 transfectant analyses. No cytokine or chemokine induction was observed with CRX-526 (6-6-6), and maximal activity was seen with CRX-527 (10-10-10). The dose-response relationship between AGP and *in vivo* chemokine induction revealed the relative potencies of CRX-
length of eight carbon atoms is necessary for TLR4 stimulation, and a length of 10 carbon atoms is optimal. Maintenance of this acyl chain length was especially critical for the secondary fatty acid on the left side of the lipid A structure. The responses of these compounds in vitro using cell-based assays and TLR4 transfectants strongly correlated with their in vitro activity.

The development of the AGP family of lipid A mimetics was based on our previous observations with monophosphoryl lipid A (MPL® adjuvant), a detoxified version of LPS from Salmonella minnesota widely used as an immunological adjuvant in human vaccines (6). MPL® adjuvant, a product resulting from sequential acid and base hydrolysis of native S. minnesota LPS, is a mixture of multiple congeners including hexa-, penta-, tetra-, and triacylated species. The most active pharmacophore was determined to be a hexa-acylated species in both human and rodent studies. These data are in agreement with the results from Hajjar et al. (32) in which natural hexa-acylated species of Pseudomonas lipid A stimulated the strongest inflammatory response from human TLR4-positive cells compared with less acylated lipid A moieties. Interestingly that group also reported the induction of several inflammatory cytokines in human peripheral blood mononuclear cells by triacylated monosaccharide lipid A-like compounds. It is tempting to speculate that the macro-molecular structure of these compounds in solution mimics the conical hexa-acylated structure thought to be associated with TLR4 agonist activity (34).

Within a defined family of synthetic hexa-acylated compounds with an identical backbone structure, we were able to identify a strict dependence on secondary acyl chain length and TLR4 agonist activity. Specifically a secondary acyl chain length of eight carbons or more was necessary to elicit any inflammatory response, and a length of 10 carbons was optimal relative to longer acyl chains of 12 or 14 carbons. Within the optimal 10-carbon framework, substitutions of six-carbon acyl groups identified a hypervariable region specific to human TLR4 itself that confers the ability to discriminate between penta-acylated and hexa-acylated lipid A species. Consistent with these data, the synthetic hexa-acylated species used in our studies were more potent than the naturally derived MPL® adjuvant and a limited number of synthetic penta-acylated AGP compounds generated (data not shown). Recently Tamai et al. (33) reported TLR4-specific antagonist activity in vitro using a synthetic hexa-acylated disaccharide molecule based on the structure of E. coli lipid A but not the reduced tetra-acylated species. Curiously that group also reported the induction of several inflammatory cytokines in human peripheral blood mononuclear cells by triacylated monosaccharide lipid A-like compounds. It is tempting to speculate that the macro-molecular structure of these compounds in solution mimics the conical hexa-acylated structure thought to be associated with TLR4 agonist activity (34).

We observed that the results of the microarray analysis from the AGP-stimulated monocytes most closely mirrored the data from the TLR4/MD-2 transfectants. Specifically both systems were able to detect weak or partial agonist activity (e.g. with CRX-565 (10-6-10)) that was not as readily apparent when TNF-α was analyzed from stimulated macrophages. The clustering analysis from the microarray data highlighted the fact that the synthetic TLR4 agonists induce a transcriptional response nearly identical to that of native LPS. Comparing the transcriptional responses across the family of compounds suggested that the weaker agonists induced a qualitatively similar response to the stronger agonists or LPS, i.e. reduced levels and/or a limited subset of genes induced. While the data presented herein reflect a highly selected group of target genes, further analysis using microarrays in which more than 1000 “unselected” genes induced by LPS were arrayed as targets have revealed the same pattern. Interestingly the results from the various in vitro analyses correlated with the biological potency of the respective molecules in vivo in the context of chemokine induction.

The intended application for MPL® adjuvant is as an immunological vaccine adjuvant, which would potentiate both B and T cell responses to co-administered antigen. Indeed MPL® adjuvant has been administered to more than 12,000 humans in the context of numerous infectious disease and cancer vaccine trials. Similarly the “prototype” AGP, RC529 (6), has been used successfully in a Phase III clinical trial combined with a conventional alum-based hepatitis B vaccine. More recently, we have been able to demonstrate the ability of members of the AGP family to confer protection against Listeria or influenza virus challenge in the absence of co-administration of the microbe itself or microbial antigen. Taken together, the potent chemokine/cytokine induction and ability to confer protection against infectious challenge in the absence of antigen highlight the possibility that TLR4 agonists may be used as stand-alone agents in infectious and/or atopic/allergic disease. The success of synthetic TLR7 agonists (imiquimod/R-848) (35) and TLR9 agonists (CpG oligonucleotides) (36) as stand-alone immunomodulators in clinical development provides a clear precedent for this approach and paves the way for TLR4-based immunomodulatory approaches. Regardless these synthetic compounds have proved helpful in elucidating structure-function relationships relevant to the interaction between lipid A and TLR4 and will prove to be useful compounds in a variety of cell-based and animal studies investigating TLR4.

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Structure-Activity of Synthetic TLR4 Agonists

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Axel G. Stöver, Jean Da Silva Correia, Jay T. Evans, Christopher W. Cluff, Mark W. Elliott, Eric W. Jeffery, David A. Johnson, Michael J. Lacy, Jory R. Baldridge, Peter Probst, Richard J. Ulevitch, David H. Persing and Robert M. Hershberg

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