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Phosphoproteome and transcription factor activity profiling identify actions of the anti-inflammatory agent UTL-5g in LPS stimulated RAW 264.7 cells including disrupting actin remodeling and STAT-3 activation

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Abstract

UTL-5g is a novel small-molecule TNF-alpha modulator. It reduces cisplatin-induced side effects by protecting kidney, liver, and platelets, thereby increasing tolerance for cisplatin. UTL-5g also reduces radiation-induced acute liver toxicity. The mechanism of action for UTL-5g is not clear at the present time. A phosphoproteomic analysis to a depth of 4943 phosphopeptides and a luminescence-based transcription factor activity assay were used to provide complementary analyses of signaling events that were disrupted by UTL-5g in RAW 264.7 cells. Transcriptional activity downstream of the interferon gamma, IL-6, type 1 Interferon, TGF- β , PKC/Ca²⁺ and the glucocorticoid receptor pathways were disrupted by UTL-5g.

Phosphoproteomic analysis indicated that hyperphosphorylation of proteins involved in actin remodeling was suppressed by UTL-5g (gene set analysis, FDR < 1%) as was phosphorylation of Stat3, consistent with the IL-6 results in the transcription factor assay. Neither analysis indicated that LPS-induced activation of the NF- κ B, cAMP/PKA and JNK signaling pathways were affected by UTL-5g. This global characterization of UTL-5g activity in a macrophage cell line discovered that it disrupts selected aspects of LPS signaling including Stat3 activation and actin remodeling providing new insight on how UTL-5g acts to reduce cisplatin-induced side effects.

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Supplementary data. Protein identification and localization and quantitative data for phosphopeptides identified in this study.

Keywords

UTL-5g; LPS; phosphoproteomics; mass spectrometry; anti-inflammatory; macrophage

1. Introduction

UTL-5g is a novel small-molecule TNF- α modulator with several beneficial pharmacologic effects. For example, UTL-5g reduces cisplatin-induced toxicity by protecting kidney, liver, and platelets, thereby increasing the tolerance of mice for cisplatin (Shaw et al., 2013). UTL-5g increases the survival rates of mice treated with lipopolysaccharide (LPS) (Zhang et al., 2014) and reduces radiation-induced liver damage (Shaw et al., 2012). Given the critical role for macrophages in inflammation we hypothesized that UTL-5g exerts a primary anti-inflammatory effect *in vivo* by suppressing macrophage activation. In support of this, UTL-5g inhibits LPS-stimulated PGE2 production in mouse RAW 264.7 cells by more than 50% (Shaw, 2015). In addition, an analog of UTL-5g blocks LPS induced NO production in RAW 264.7 cells (Shaw et al., 2011). No studies have examined the mechanism by which UTL-5g disrupts those inflammatory processes.

LPS initiated signaling is required for the immunological response to Gram negative pathogens. LPS from many bacterial species will initiate acute inflammatory responses in mammals that are typical of the host reaction to infection and immune cell responses to LPS exposure is a tool to investigate immune responses (Xie et al., 1994). The mouse RAW 264.7 cell line, derived from macrophage/monocyte tumor cells, is a common model for studying LPS-induced inflammation. RAW 264.7 cells produce a battery of mediators and proinflammatory cytokines when exposed to LPS, and the paradigm of LPS treatment of cultured RAW cells is used extensively to investigate the mechanisms of action for anti-inflammatory compounds (Chiang et al., 2005; Kim et al., 2007).

Genome- and system-scale technologies are valuable tools for mechanism discovery as they enable novel and unanticipated findings (Coombs et al., 2012). The expanding capabilities of mass spectrometry based phosphoproteomics in terms of depth of coverage and sample multiplexing (Erickson et al., 2015; Sharma et al., 2014), are making it a powerful systems-level approach for mechanism of action studies. It has already been applied to mechanism of action determination for inorganic mercury (Caruso et al., 2014; Carruthers et al., 2014), deoxyvinylol (Pan et al., 2013), ammonia (Harder et al., 2014), and to identify the target profiles of kinase inhibitors (Li et al., 2010; Pan et al., 2009). It has emerged from these studies and from other investigations of compounds with known mechanisms (Pines et al., 2011) that the phosphoproteome can provide a more precise determination of mechanism of action than transcriptome or total proteome analysis. Further, because LPS signaling is mediated by a well characterized cascade of protein phosphorylation, phosphoproteomic analysis is a natural choice to investigate disruptions of its action.

A major outcome of LPS stimulation of RAW 264.7 cells is an increase in transcription of genes whose products mediate inflammatory responses. In order to capture both the dynamics of the phosphorylation cascade and its transcriptional outcome, which comprise the main components of LPS signaling in macrophages, we applied a combination of

phosphoproteomics and transcription factor activity analysis to examine the mechanism by which UTL-5g suppresses LPS activation of RAW cells.

2. Materials and Methods

2.1 Chemicals and Reagents

Lipopolysaccharide (LPS, *E. coli* 0111:B4) was obtained from Sigma-Aldrich (St. Louis, MO). M-PER protein extraction reagent, phosphatase inhibitors and Opti-MEM® were purchased from Thermo-Fisher Scientific (Waltham, MA). Dulbecco's modified eagle medium (DMEM), bovine calf serum (BCS), fetal bovine serum (FBS), non-essential amino acids (NEAA) and penicillin/streptomycin were obtained from (HyClone, Logan, UT). Cignal Finder Immune Signaling Pathway Reporter Arrays and Attractene transfection reagent were products of QIAGEN (Valencia, CA). Dual-Luciferase reporter assay system was purchased from Promega (Madison, WI). RAW (264.7) mouse macrophage/monocytic cell line was acquired from ATCC (Manassas, VA). They were routinely grown in DMEM supplemented with 10% heat inactivated BCS. Anti-p-JNK (T183/Y185) rabbit polyclonal antibody and peroxidase-labeled goat anti-rabbit secondary antibody were obtained from R&D Systems (Minneapolis, MN). BCA protein assay reagent was purchased from Pierce (Waltham, MA). TiO₂ beads were obtained from Titansphere, GL Sciences (Tokyo, Japan). Tandem mass tag labeling reagents were obtained from Thermo Fisher Scientific (Waltham MA). Dithiothreitol (DTT), iodoacetamide (IAA) and Lithium dodecyl sulphate (LiDS) were purchased from Sigma (St Louis, MO). UTL-5g (Lot#1182-MEM-3D, Purity > 99%) (Fig. 1) was synthesized at Kalexsyn Medicinal Chemistry (Kalamazoo, Michigan). The polyclonal anti-p-Ser5 L-plastin antibody was a kind gift from Dr. Elisabeth Schaffner-Reckinger (University of Luxembourg) (Janji et al., 2006).

2.2 Transcription Factor Assay

Multi-pathway activity assays were carried out using Cignal Finder Immune Signaling 10-Pathway Reporter Arrays according to the manufacture's instruction. Transfection of RAW 264.7 cells (1×10^5 /well) was performed using Attractene (0.4 μ l/well) at 37°C for 16 h. After transfection, cells were washed and replenished with assay medium (Opti-MEM® containing 0.5% of FBS, 1% NEAA, 100 U/ml penicillin and 100 μ g/ml streptomycin). Thereafter, cells were treated with varying doses of UTL-5g for 60 min and then challenged with 100 ng/ml of LPS. After an additional 16 h of incubation, cells were washed and lysed. Luciferase assay was carried out with Dual-Luciferase reporter assay system and the assay was performed directly in the multi-well plate with Fluoroskan FL microplate luminometer (Thermo Fisher Scientific) at room temperature.

2.3 Western Blot analysis

For the JNK analysis, RAW 264.7 cells (2×10^6 /well) were treated with UTL-5g from 0.2 to 10 μ M at 37°C for 60 min, followed with LPS 10 μ l/well (100 ng/ml final) for an additional 30 min. Thereafter, cells were washed once in cold MEM and lysed with 150 μ l of M-PER protein extraction reagent containing protease and phosphatase inhibitors and EDTA. The cell mixtures were vortexed gently for 10 min on ice and cell debris were removed by centrifugation at $14,000 \times g$ for 15 min. The sample supernatants were removed and

separated by SDS-PAGE (12% gel). After transfer to a nitrocellulose membrane, the membrane was blotted with anti-p-JNK (Thr183/Tyr185) rabbit polyclonal antibody followed by peroxidase-conjugated goat anti-rabbit secondary antibody. JNK bands were visualized by enhanced chemiluminescence detection methods.

For the plastin-2 analysis RAW 264.7 cells (2×10^6 /well) were treated with 10 or 50 μ M UTL-5g at 37°C for 60 min, followed with LPS 5 μ l/well (50 ng/ml final) for an additional 30 min. Samples were processed as indicated for the phospho JNK analysis except that the nitrocellulose membrane was probed with anti-p-plastin (Ser5).

2.4 Sample Preparation and LC-MS3

RAW 264.7 cells (1.0×10^6 /dish) were grown for three days in 15 cm dishes, reaching 80% confluence ($>3 \times 10^7$ /dish) and then treated as described in the results (section 3.2). An additional sample treated with 25 μ M pervanadate for 15 min was also prepared to improve our ability to detect phosphotyrosine (pTyr). Pervanadate treatment can result in an accumulation of pTyr to up to 20% of phosphorylated residues (Caruthers et al., 2014). We reasoned that the inclusion of a pTyr enriched sample would improve our ability to detect pTyr residues in data-dependent LC-MS3 analysis. The dishes were placed on ice and harvested by scraping and then rinsed with ice-cold Hank's solution, pelleted and frozen. Cells were lysed by resuspension in 1% LiDS and heating at 95°C for 5 min. Protein concentration in the lysates was determined using a BCA assay. Samples were reduced with DTT and alkylated with IAA and then digested overnight with trypsin at 1:50 (w/w) at a sample protein concentration of 1.0 mg/ml in buffer containing 100 mM Tris, 0.1% LiDS, and 10% acetonitrile. All samples were evaluated by SDS-PAGE to ensure full digestion before proceeding to phosphopeptide isolation. Phosphopeptides were isolated using 5 μ m TiO_2 beads at a ratio of beads to protein of 8:1 (w/w). Eluted peptides were dried, resolubilized and evaporated again to remove ammonium from the TiO_2 procedure. Samples were then resolubilized in buffer for TMT labeling, which was carried out according to the manufacturer's instructions. The individual samples were then pooled for analysis. The pooled, labeled peptides were fractionated using an SCX MicroSpin Column (Harvard Apparatus, Holliston MA) with elution in 7 fractions using 5 to 125 mM ammonium formate. Each fraction was dried, resolubilized in 0.1% FA and submitted for LC/MS3 analysis. Peptides were loaded onto a 75 μ m $\times$ 25 cm, Acclaim PepMap 100 column and eluted into a Thermo Fusion Tribrid mass spectrometer (Thermo Fisher Scientific) by a gradient of acetonitrile from 2 to 30% over 110 min. The mass spectrometer was set to conduct multistage MS3 analysis to limit interference from coeluting peptides (McAlister et al., 2014). The top 10 most abundant peptides in each MS1 scan were selected for MS2 fragmentation using CID at 32 to obtain sequencing ions and MS3 fragmentation of the top 10 MS2 peaks using HCD at 55 to obtain reporter ions for quantitation. Following the initial analysis, each fraction was analyzed again under the same conditions but all non-phosphorylated peptides identified in the first analysis were excluded from analysis.

2.5 Spectra-Peptide Matching

Mass spectra were searched using MaxQuant version 1.5.2.8 (Cox and Mann, 2008) against the Uniprot mouse database (downloaded 2014.06.24; 16,670 sequences). Database matches

for proteins and peptides were accepted at a 1% false discovery rate. Phosphorylation of Ser, Thr and Tyr residues, oxidation of Met and protein N-terminal acetylation were specified as variable modifications and carbamidomethylation of Cys was a fixed modification. The minimum score and minimum delta score for identification were set to 0 for all peptides. Phosphorylation site localization was accepted if the confidence was greater than 80%.

2.6 Bioinformatic Analysis

Analysis of MaxQuant output was conducted in R version 3.1.3 (R_Core_Team, 2015). We constructed ratios for each relevant pairwise treatment comparison, $\log_2$ transformed those ratios and set their medians to 0. Technical variability is increased for low intensity reporter ions (Karp et al., 2010). Therefore, we converted ratios to Z-scores using an intensity-local measure of variability. $\log_2$ ratios for each phosphopeptide were scaled to their local median absolute deviation (MAD). The local MAD was a rolling average of $\log_2$ ratios ordered by intensity that was then smoothed using LOWESS. An absolute Z-score greater than 1 was accepted as an indication of a treatment effect. All conclusions based on this criterion are supported by orthogonal data including western blots or luciferase assays.

Reactome pathway gene set analysis was conducted using PIANO (Varemo et al., 2013). This software allows the submission of multiple instances of the same protein in a dataset so that proteins with multiple phosphopeptides don't have to be collapsed to a single quantitative value. Reactome gene sets were downloaded from the Bader lab website (<http://baderlab.org/GeneSets>, accessed April 24, 2016) (Merico et al., 2010). Phosphopeptide Z-scores annotated with Uniprot accession numbers were submitted for analysis. The GSEA statistic was used to identify enriched sets and gene sets were required to have between 10 and 100 members in our data to be analyzed. Reactome pathways that were affected by LPS vs untreated (FDR < 2%) were selected for further analysis. To reduce redundancy in identified Reactome pathways, they were pooled according to their overlap in phosphoproteins so that any pathways that shared more than 90% of the same proteins were assigned to the same pool.

For the comparison to external data, supplemental data table S1 was downloaded from Weintz et al (Weintz et al., 2010). Only primary amino acid sequences were used and information about the site and number of phosphorylations was discarded. The PIANO package was also used to validate the overlap of phosphopeptides that responded to LPS in datasets from Weintz et al (Weintz et al., 2010) and from this current study. Peptide sequences that Weintz et al annotate as up-regulated at 15 min were used as a set and compared to our data using the GSEA statistic.

2.7 Protein Names

All proteins identified by mass spectrometry in this experiment are referred to by their full names or their gene names according to Uniprot.

3. Results

3.1 Transcription Factor Activity

Exposure of RAW 264.7 cells to LPS initiates a receptor mediated signaling cascade leading to increased cell motility and production of pro-inflammatory cytokines. We hypothesized that pretreatment of RAW cells with UTL-5g (Fig. 1) would suppress the LPS induced signaling cascade at a limited number of nodes in the pathway. We further expected that the UTL-5g pretreatment would reduce LPS-stimulated hyperphosphorylation or transcription factor activation in a way that would reflect on the site or sites of UTL-5g activity.

A panel of luciferase reporter assays was used to query 10 transcription factors that are downstream of 10 different immune related signaling pathways. RAW 264.7 macrophages were transfected with the respective reporter assay plasmids, pre-treated with UTL-5g at 1, 10 or 50 μ M for 60 min and then challenged with 100 ng/ml LPS. After a 16 h incubation, transcription factor activity was measured. Transcription factors that showed a UTL-5g dose-dependent decrease in activity in two experiments were categorized as being disrupted by UTL-5g. Surprisingly, the NF- κ B, cAMP/PKA and C/EBP signaling pathways were not affected by UTL-5g. Those pathways, which activate Cox-2 gene expression (Wadleigh et al., 2000), are common targets for anti-inflammatory compounds (Chiang et al., 2005; Kim et al., 2007). Type-1 interferon signaling was also unaffected by UTL-5g. In contrast, signaling by the interferon gamma, IL-6, type 1 Interferon regulation, TGF- β , PKC/Ca²⁺ and the glucocorticoid receptor pathways were decreased by UTL-5g pretreatment suggesting they could be targets for UTL-5g (Fig. 2). Because signaling by the Jun N-terminal Kinase (JNK) pathway is relevant for LPS signaling but was not tested using the luciferase assay, we measured its activity indirectly using western blot. Western blot analysis indicated that UTL-5g had no effect on LPS-stimulated JNK phosphorylation (not shown).

3.2 Phosphoproteomic Analysis Overview

To validate and expand on the transcription factor activity profile, we conducted a phosphoproteomic analysis of LPS stimulated RAW cells with or without UTL-5g pretreatment.

RAW 264.7 cells were pretreated with 50 μ M UTL-5g or vehicle (DMSO) for 1 h. This was followed by stimulation with 100 ng/ml LPS or vehicle for 15 min prior to harvest. Identical treatment conditions cause a greater than 50% inhibition of LPS-induced PGE2 production (Shaw, 2015). Phosphopeptides were extracted from treated cells and analyzed as described in the materials and methods. This study identified 4943 phosphopeptides and 2784 unique phosphoproteins. 4702 phosphorylation sites could be localized with greater than 80% confidence. Those sites included 2832 phosphoserine (pSer), 1329 phosphothreonine (pThr) and 541 pTyr sites (60.2, 28.2, and 11.5% of the total sites respectively). All phosphopeptide data are included in supplementary Table 1.

3.3 The Global Impact of UTL-5g

LPS exposure stimulates protein hyperphosphorylation at hundreds of sites (Weintz et al.). If UTL-5g completely blocks LPS signaling, the phosphoproteome of samples treated with

UTL-5g and LPS would be expected to be similar to that of untreated cells. To test the global action of UTL-5g we compared samples using the percentage of phosphopeptides with a greater than 2-fold change between them as a measure of dissimilarity. 573 phosphopeptides (15.3% of quantified phosphopeptides, Fig. 3) had a greater than 2-fold increase in abundance in response to between LPS exposure. UTL-5g pretreatment produced a small increase to 684 (18.7%) in the number of phosphopeptides that had a greater than 2-fold change in response to LPS exposure. In contrast UTL-5g and LPS treated cells were similar to LPS treated cells alone as 190 phosphopeptides (5.0%) were more than 2-fold different between the two samples. These data indicate that UTL-5g does not globally block LPS signaling but rather disrupts selected pathways.

3.4 Core LPS Signaling Pathways

We used gene set analysis (Varemo et al., 2013) to identify pathways that were selectively affected by LPS and UTL-5g. Reactome signaling pathways (Croft et al., 2014) were tested for enrichment among hyperphosphorylated or dephosphorylated phosphopeptides. Phosphopeptides that were hyperphosphorylated in response to LPS treatment were enriched in expected pathways including Innate Immune System, Toll Like Receptor 4 (TLR4) cascade and MAP kinase activation in TLR cascade (FDR < 1%). UTL-5g pretreatment didn't cause a significant change in phosphorylation of peptides in those pathways relative to LPS alone suggesting that UTL-5g didn't affect LPS signaling through those pathways. Manual examination of phosphopeptides in those pathways confirmed that for the vast majority of them LPS induced hyperphosphorylation wasn't suppressed by UTL-5g. To identify pathways that were suppressed by UTL-5g, 2 criteria were used. Signaling pathways had to be (1) enriched among LPS hyperphosphorylated peptides and (2) enriched in peptides that are dephosphorylated in LPS+UTL-5g treated samples relative to LPS alone. We identified 2 sets of pathways that met those criteria: 3 related to G2-M transition and 2 related to phagocytosis and actin remodeling (Fig. 4). Pathways associated with G2-M transition, phagocytosis and actin remodeling and heat stress response clusters have significantly lower phosphorylation in LPS+UTL-5g vs LPS treated cells (FDR < 1%).

3.5 Comparison to External Data

We compared our dataset to data generated by a study by Weintz *et al* of LPS signaling in primary cultured macrophages (Weintz et al., 2010) to benchmark our results and validate our identification of LPS responding phosphopeptides. Their experiment included a treatment with 100 ng/ml LPS for 15 min which is identical to the treatment used here. Weintz *et al* reported 4432 unique phosphopeptide sequences, 774 of which they considered upregulated after LPS treatment. We identified 1110 of the phosphopeptides found in Weintz *et al* and 212 of the hyperphosphorylated ones (Fig. 5). The correlation between the two datasets was modest ($R^2=0.15$), however phosphopeptides that were hyperphosphorylated according to Weintz *et al* were shifted towards higher Z-scores (FDR < 0.01%, PIANO). We reasoned that in spite of difference between the cell systems examined in the study of Weintz *et al* and in our study many aspects of the LPS signaling cascade would be the same. Therefore, we could have increased confidence that phosphopeptides identified in both studies as responding to LPS are contributing to LPS signaling cascades. Of the 212 hyperphosphorylated peptides from Weintz *et al* that we identified, 102 had an absolute Z-

score >1 in our study and were considered the most confident targets of LPS-induced hyperphosphorylation. Those phosphopeptides were evaluated for a response to UTL-5g pretreatment. Phosphopeptides were ranked according to their Z-score for LPS+UTL-5g vs LPS alone from lowest (most dephosphorylated) to highest to find those phosphopeptides whose LPS-induced hyperphosphorylation were most strongly blocked by UTL-5g (the top 10 are shown in Table 1). Of the top 10 proteins, 5 are involved in actin rearrangement and membrane dynamics: plastin-2, Disabled homolog 2, Golgi reassembly-stacking protein 2, Dynamin-1-like protein, and Paxillin. This is in agreement with the ontology analysis (Fig. 4) that determined that pathways involved in actin remodeling were disrupted by UTL-5g pretreatment. Previous research corroborates the hyperphosphorylation of Ser5 of plastin-2 in response to LPS. Its phosphorylation regulates actin cytoskeleton assembly (Janji et al., 2006; Shinomiya et al., 1995; Wabnitz et al., 2007). The disruption of LPS-induced phosphorylation of Ser5 of plastin-2 by UTL-5g was confirmed by western blot analysis (Fig. 6).

3.6 Agreement Between Phosphoproteomics and Transcription Factor Activity

To test whether the phosphoproteomic and transcription factor analyses were concordant, we examined our data for phosphorylation sites that could regulate the transcription factors tested in the luciferase assay. Our analysis was restricted to transcription factors that had either Reactome (Croft et al., 2014) pathway data regarding their activity or Uniprot annotated sites that bore directly on transcription factor activity. In addition, phosphorylation sites had to have an absolute Z- score >1 for at least one of the LPS vs control or LPS +UTL-5g vs LPS comparisons to be considered for this analysis.

NF- κ B transcriptional activity was not affected by UTL-5g (Fig. 2). In agreement with the transcription assay, the Reactome pathway TRAF6 mediated induction of NF- κ B and MAP kinases (Reactome 9181570) showed an indistinguishable change due to LPS treatment regardless of UTL-5g (not shown). NF- κ B activity is regulated by the abundance of the inhibitory proteins I κ B and by phosphorylation of p65. LPS stimulation of NF- κ B activity via I κ B degradation is mediated by the MyD88/TRAF6 signaling pathway, which includes the I κ B kinases I κ BKA and I κ BKB(Ikbbk)(Guha and Mackman, 2001). Ser 672 of Ikbbk underwent hyperphosphorylation in response to LPS treatment which was decreased by UTL-5g pretreatment (Fig. 7A). This residue is part of the C-terminal Serine cluster that is autophosphorylated by activated Ikbbk and attenuates activity (Delhase et al., 1999). In addition, three phosphopeptides were identified from Nfkb2 itself, spanning the amino acid residues 413–442, 412–442 and 425–442. For each of those phosphopeptides, the raw reporter ion intensities in LPS+UTL-5g treated samples were greater than or equal to the intensities in LPS treated samples (8529 vs 7930, 7931 vs 6406 and 3781 vs 3392 respectively), suggesting that the protein abundance of Nfkb2 was not decreased in the UTL-5g pretreated samples in agreement with its transcriptional activity.

Stat3-regulated transcriptional activity was decreased by UTL-5g pretreatment (Fig. 2). Ser727, which is essential for full activity of Stat3 (Wen et al., 1995) was hyperphosphorylated by LPS treatment relative to control and this hyperphosphorylation

was blocked by UTL-5g (76% reversal, LPS+UTL-5g vs LPS, Fig. 7B). This is consistent with the transcription factor activity assay (IL-6 in Fig. 2).

Atf2 is a transcription factor that binds to the cAMP response element (CRE, cAMP in Fig. 2). We detected phosphorylation at three phosphorylation sites. One, Thr53 is noted by Uniprot as a mediator of increased transcriptional activity and its phosphorylation was unaffected by UTL-5g, in agreement with the lack of response of CRE directed transcriptional activity. Ser94 of Atf2 had a Z-score less than -1 for the comparison between LPS and LPS+UTL-5g (Fig. 7C). However its phosphorylation does not, to our knowledge, affect the activity of ATF2 and so is not considered in this analysis. The other members of the transcription factor activity panel were not captured by the phosphoproteomic analysis.

The JNK signaling pathway was not represented in the transcription factor analysis. However JNK phosphorylation on its activation loop (Thr185/Tyr187) was measured by western blot (not shown) and Jun pSer73 as well as other JNK substrates were quantified in the phosphoproteomic analysis. JNK phosphorylation as well as that of its substrates Jun Ser73, Raptor Ser863 (Fig. 7D) and Atf2 Thr53 (Fig. 7C) were stimulated by LPS and unaffected by UTL-5g.

4. Discussion

UTL-5g is a chemoprotective agent (Shaw et al., 2010; Shaw et al., 2013) that eases the chemotherapy-induced inflammation that can limit the dose and duration of treatment. We previously reported that UTL-5g blocks LPS-stimulated PGE2 production in the mouse RAW 264.7 cell line (Shaw, 2015) validating LPS treated RAW 264.7 cells as a model system for investigating the mechanism for UTL-5g anti-inflammatory activity. Using a combination of transcription factor activity and phosphoproteomic analyses to interrogate this model system we identified several aspects of LPS signaling that were disrupted by UTL-5g including inhibition of Stat3 signaling and cytoskeletal remodeling.

Macrophage activation initiates cytoskeletal remodeling that is required for phagocytosis and cell migration. Our phosphoproteomics analysis indicated that phagocytosis and actin remodeling Reactome pathways were 1) among the most hyperphosphorylated pathways in LPS stimulated RAW cells and 2) among the most dephosphorylated pathways when UTL-5g treatment preceded LPS (Fig. 4). Further, in a complementary analysis of our data using phosphopeptides identified in Weintz *et al* (Weintz et al., 2010) to confirm LPS-induced hyperphosphorylation the n-terminal phosphopeptide of platin-2 containing Ser5 was the most strongly blocked phosphorylation by UTL-5g (Table 1). The UTL-5g sensitivity of platin-2 pSer5 hyperphosphorylation by LPS was confirmed by western blot (Fig. 6). Platin-2 phosphorylation at Ser5 is a hallmark of this macrophage cytoskeleton remodeling (Janji et al., 2006; Shinomiya et al., 1991). Phosphorylation at this site activates the actin-bundling activity of platin-2 resulting in the formation of cellular protrusions (Janji et al., 2006). Platin-2 is required for the full activation of T-cells (Wang et al., 2010) and further is a target for dexamethasone that is independent of its effect on transcription (Wabnitz et al., 2011) indicating that it is possible for platin-2 phosphorylation to participate in cellular signaling. However no role for platin-2 phosphorylation in the release

of inflammatory mediators in macrophages has been established and further research will be required to determine the relationship between the disruption of platin-2 phosphorylation by UTL-5g and its disruption Stat3 activation and PGE2 production. It is also notable that cancer progression correlates with platin-2 level (Otsuka et al., 2001) and that platin (Otsuka et al., 2001) overexpression can induce proliferation and invasion in cancer cells (Foran et al., 2006). These findings raise the possibility that UTL-5g could make a two-pronged contribution to anti-cancer treatment: reducing treatment induced inflammation and reducing tumor cell proliferation and invasion.

An interconnected network of signaling events mediate the LPS-induced production of inflammatory mediators including PGE2. Anti-inflammatory molecules that disrupt PGE2 production commonly disrupt the activity of the inducible enzyme COX2 that catalyzes the rate limiting step in its production. COX2 transcriptional activation is primarily mediated by C/EBP family transcription factors and c-Jun (Wadleigh et al., 2000) however our results suggest that neither of those pathways were affected by UTL-5g. Hyperphosphorylation at c-Jun S73, a JNK substrate site was induced by LPS and unaffected by UTL-5g (Fig. 7D). Similarly JNK phosphorylation at T183/Y185 was stimulated by LPS and unaffected by UTL-5g (not shown). C/EBP transcriptional activity was also unaffected by UTL-5g (Fig. 2). Taken together these results indicate that UTL-5g doesn't disrupt the signaling pathways and transcription factors commonly associated with COX2 transcriptional activation and further work will be required to elucidate the link between UTL-5g and reduced PGE2 production.

The transcription factor activity and phosphoproteomic analyses are a powerful combination for characterizing anti-inflammatory drug effects because they provide complementary measures of signaling system activation. The phosphoproteomic data, collected after 15 min of LPS treatment captured early LPS-stimulated signaling events while the transcription factor data collected after 16 h captured the downstream events. Careful scheduling of the time points for data collection was essential for this analysis as LPS-stimulated protein phosphorylation predominately occurs before 1 h while the transcription factor response has a more variable onset and can be sustained for 24 h (Matsuzawa et al., 2005; Noman et al.). The transcription factor analysis found that NF- κ B, cAMP/PKA and C/EBP signaling pathways were not disrupted by UTL-5g while the interferon gamma, IL-6, type 1 Interferon regulation, TGF- β , PKC/Ca²⁺ and the glucocorticoid receptor pathways were. The selective action of UTL-5g on transcriptional activity was in agreement with a global interpretation of the phosphoproteomic data where the UTL-5g+LPS phosphoproteome was more similar to LPS alone than it was to untreated (Fig. 3). More specifically, data from both assays were collected regarding the cAMP/PKA, NF- κ B and Stat3 signaling pathways and in all cases were in agreement. ATF2 phosphorylation at Thr53 (Fig. 7C) and transcriptional activity (cAMP/PKA Fig. 2) were both unaffected by UTL-5g. NF- κ B transcriptional activity was not affected by UTL-5g (Fig. 2) while the phosphoproteomics results corroborated this by indicating that Nfkb2 abundance was also unaffected by UTL-5g. Finally, LPS-stimulated Stat3 phosphorylation at Ser727 was blocked by UTL-5g, consistent with the decrease in Stat3 transcriptional activity in UTL-5g treated cells (IL-6, Fig. 2). The mutual confirmation of decreased Stat3 activity in UTL-5g treated cells suggests that it is a potential target of UTL-5g. Stat3 is a target of several other compounds with anti-inflammatory activity

including curcumin (Bharti et al., 2003) and celecoxib (Liu et al., 2011), suggesting that Stat3 may be a key target molecule in the anti-inflammatory activity of UTL-5g.

The phosphoproteome and transcription factor activity analyses jointly support the unexpected conclusion that despite a robust suppression of LPS stimulated PGE2 production by UTL-5g, it had virtually no effect on JNK, NF- κ B and cAMP/PKA signaling. We have identified actin remodeling and Stat3 signaling as potential targets for UTL-5g anti-inflammatory activity. Thus, the activity of UTL-5g may be mediated through a NF- κ B independent signaling pathway. Further work will be required to understand the role of those targets in the physiological response to UTL-5g.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Chemical structure of UTL-5g

Fig. 2.

A panel of 10 luciferase-based transcription factor reporter assays were used to investigate signaling pathway inhibition by UTL-5g. Each set of 4 bars indicate luciferase intensity from left to right of cells treated with LPS alone or LPS + 1, 10 or 50 μM UTL-5g. The **NF-κB** indicates NF-κB transcriptional activity, **Type 1 Interferon** indicates STAT1/STAT2 activity, **interferon gamma** indicates STAT1/STAT1 activity, **IL-6** indicates STAT3 activity, **Interferon Regulation** indicates interferon regulatory factor 1 activity, **TGF-β** indicates SMAD2/SMAD3/SMAD4 transcriptional activity, **cAMP/PKA** indicates CREB activity, **PKC/Ca⁺⁺** indicates NFAT activity, **C/EBP** and **Glucocorticoid Receptor** indicate the activity of those transcription factors. Error bars indicate standard deviation, n=2. The data presented are representative of duplicate assays.

Fig. 3.

Phosphopeptides in RAW 264.7 macrophages treated with LPS, UTL-5g or both, along with a vehicle treated control were quantified using TMT-labeling and LC-MS3. The proportion of phosphopeptides that changed more than two-fold between samples is shown as a measure of sample dissimilarity. 21.5% of phosphopeptides changed by more than 2-fold between LPS and vehicle treated samples, 23.9% between LPS+UTL-5g and UTL-5g and 11.2% between LPS and LPS+UTL-5g.

Fig. 4.

Identification of Reactome Pathways whose hyperphosphorylation by LPS is blocked by UTL-5g. Reactome Pathways that were hyperphosphorylated by LPS relative to vehicle treatment (gene set analysis, 1% FDR) were ranked according to their FDR corrected p-value for hyperphosphorylation in LPS vs control cells (x-axis) and for dephosphorylation in LPS+UTL-5g treated samples vs LPS alone (y-axis). The most significant pathways are ranked 1. Pathways were clustered into groups to reduce redundancy and clusters were given a descriptive name. The median value for each cluster is plotted.

Fig. 5.

For phosphopeptides identified in our experiment and in Weintz *et al* their log2-fold changes for a 15 min LPS vs control treatment are plotted against our Z-scores for LPS vs untreated. Phosphopeptides that responded to LPS according to Weintz *et al* are highlighted in red. The horizontal lines indicate a 2-fold change and the vertical lines indicate Z-scores of positive and negative 1

Fig. 6.

A western blot was used to quantify L-plastin phosphorylation at Ser5. Cells were untreated or treated with 50 ng/ml LPS, 50 μ M UTL-5g, or UTL-5g followed by LPS. Actin quantified as a loading control. Normalized band density quantifications are above their respective bands. This blot is representative of 2 experiments.

Fig. 7.

Reporter ion intensities for phosphorylation sites that were relevant to transcription factor activities reported in Fig. 1. The pathways represented are (A) NF- κ B, (B) IL-6, (C) cAMP and (D) JNK-AP1. Error bars indicate the range of values corresponding to a Z-score of 1 or less as calculated for LPS+UTL-5g vs LPS. * indicates sites where the Z-score for LPS +UTL-5g vs LPS is less than -1 indicating a UTL-5g affected site. n=1.

Table 1
Phosphopeptides whose LPS-induced hyperphosphorylation was blocked by UTL-5g.

Gene Name	Position	Sequence	Most likely phospho-site	Z-score, LPS+UTL-5g vs LPS	Intensity		
					Unt ^a	LPS	LPS+UTL-5g
Lcp1	4–15	GSVSDEEMMELR	S5	−8.61	7848	50518	29027
Dab2	208–232	LGVDQMDFGDMSTPPDLNSPTESK	S227	−6.01	2334	8160	4757
Gorasp2	213–228	ISLPQMGTGTPPLK	T225	−5.96	15423	65893	46112
Ppp4r2	211–229	GHSDSSASESEVSLLSPVK	S226	−4.51	15284	40236	30842
Dnm1l	613–625	SKPIPIMPASPQK	S622	−4.24	6478	11377	8290
Pxn	126–147	SAEPSPTVMSSSLGSNLSELDLR	S137	−3.80	337	1664	823
Cyba	165–192	KKPSEGEAAAAGGPQVNPMPVTD EVV	S168	−2.92	1540	3745	2767
Tacc1	351–378	DGVSKPVGVGQPSDFTVQDALLDQ MSPK	S376	−2.84	1861	5622	4379
Nck1	82–103	RKPSV PDTASPADDDSFVDPGER	S85	−2.57	11159	23971	20927
Ybx1	203–232	RPQYSNPPVQGEVMEGADNQGAGE QGRPVR	S207	−2.56	1049	2663	1954

^aUntreated