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*J Immunol* published online 29 August 2014

http://www.jimmunol.org/content/early/2014/08/28/jimmunol.1401413

Supplementary Material http://www.jimmunol.org/content/suppl/2014/08/28/jimmunol.1401413.DCSupplemental

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TLR-Mediated Inflammatory Responses to *Streptococcus pneumoniae* Are Highly Dependent on Surface Expression of Bacterial Lipoproteins

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*Streptococcus pneumoniae* infections induce inflammatory responses that contribute toward both disease pathogenesis and immunity, but the host–pathogen interactions that mediate these effects are poorly defined. We used the surface lipoprotein-deficient Δ*lg* pneumococcal mutant strain to test the hypothesis that lipoproteins are key determinants of TLR-mediated immune responses to *S. pneumoniae*. We show using reporter assays that TLR2 signaling is dependent on pneumococcal lipoproteins, and that macrophage NF-κB activation and TNF-α release were reduced in response to the Δ*lg* strain. Differences in TNF-α responses between Δ*lg* and wild-type bacteria were abrogated for macrophages from TLR2- but not TLR4-deficient mice. Transcriptional profiling of human macrophages revealed attenuated TLR2-associated responses to Δ*lg* *S. pneumoniae*, comprising many NF-κB–regulated proinflammatory cytokine and chemokine genes. Importantly, non-TLR2-associated responses were preserved. Experiments using leukocytes from IL-1R–associated kinase−4–deficient patients and a mouse pneumonia model confirmed that proinflammatory responses were lipoprotein dependent. Our data suggest that leukocyte responses to bacterial lipoproteins are required for TLR2- and IL-1R–associated kinase−4–mediated inflammatory responses to *S. pneumoniae*. The Journal of Immunology, 2014, 193: 000–000.

TLR are a key mechanism for innate immune sensing and are important for the host response to *S. pneumoniae*. Genetic deficiency of TLR signaling pathway proteins substantially increases the risk of invasive *S. pneumoniae* infections (5–7). Fifty-four percent of children with IL-1R–associated kinase 4 (IRAK-4) deficiency and 41% of patients with MyD88 deficiency have at least one episode of invasive pneumococcal disease (5, 8–10). Similarly, mice deficient in the TLR-signaling molecule MyD88 are highly susceptible to *S. pneumoniae* infections (11, 12). IRAK-4 deficiency largely prevents inflammatory responses to purified TLR ligands (6, 7), including expression of the cytokines TNF-α, IL-1β, and IL-6 that are important for host immunity to *S. pneumoniae* (13–15). Although innate immune recognition of *S. pneumoniae* is dependent on contributions from several TLRs,
including TLR2, TLR4, and TLR9 (11, 16–24), the release of inflammatory cytokines such as TNF-α and IL-6 seems to be particularly dependent on TLR2 (19–21, 25). TLR2 contributes to the inflammatory response and control of infection in mouse models of meningitis and pneumonia (18, 22–24) and has additional effects that may affect disease development. First, the proinflammatory effects of TLR4 activation and of the S. pneumoniae toxin pneumolysin are partly dependent on synergistic activation of TLR2 (26, 27). Second, adaptive immune responses to S. pneumoniae can be impaired in TLR2-deficient mice (19, 28, 29). Third, TLR2-mediated respiratory epithelium responses to infection by S. pneumoniae increase tight junction breakdown and bacterial translocation across epithelial layers (30). Hence, identifying the S. pneumoniae TLR2 ligands is necessary for understanding of the molecular mechanisms contributing to disease pathogenesis by this pathogen.

Known TLR2 ligands include peptidoglycan (PGN) and lipoteichoic acid (LTA), both components of the Gram-positive cell wall (31, 32). The S. pneumoniae cell wall is highly proinflammatory, suggesting PGN and LTA could be major TLR2 agonists (33–35). However, cell wall–dependent inflammation is also mediated by NOD recognition of PGN (36, 37), and purified S. pneumoniae LTA does not stimulate IL-8 production by HEK293 cells transfected with TLR2 (38). Hence, cell wall products may not be dominant TLR2 agonists for S. pneumoniae. Bacterial lipoproteins are important TLR2 ligands for other Gram-positive pathogens (32, 39, 40), and S. pneumoniae expresses a large number of lipoproteins, many of which are important for bacterial virulence (41–46). However, the importance of S. pneumoniae lipoproteins for TLR2-mediated immune recognition and proinflammatory responses has not been investigated. We have used a S. pneumoniae mutant strain with markedly reduced surface lipoprotein content due to deletion of the lipoprotein diacylglycerol transferase gene \(\text{lgf}\) (45) to assess the contribution of lipoproteins to TLR2-dependent inflammatory responses to S. pneumoniae. The effects of lipoproteins on macrophage responses to S. pneumoniae were characterized in detail using transcriptome analysis and by measuring important proinflammatory cytokine responses of mouse macrophages with deletions affecting the TLR pathway and leukocytes from patients with IRAK-4 deficiency.

**Materials and Methods**

**Ethics statement**

Experiments using human cells were approved by the joint University College London/University College Hospitals National Health Service Trust Human Research Ethics Committee, and written informed consent was obtained from all participants. All animal experiments were approved by the University College London Biological Services Ethical Committee and the United Kingdom Home Office (Project License PPL70/6510) and performed according to United Kingdom national guidelines for animal use and care.

**Bacterial strains and growth conditions**

The S. pneumoniae strain, TIGR4, was a gift of J. Weiser (University of Pennsylvania, Philadelphia, PA). The \(\Delta_{\text{lgf}}\) strains were obtained by in-frame deletion of \(\text{lgf}\) (Sp1412) from wild-type (WT) TIGR4, D39, and \(\Delta_{\text{pab}}\) TIGR4 strains, as previously described (45, 47). Mutant strains were genome sequenced by the Wellcome Trust Centre for Human Genetics (Oxford, U.K.) using an Illumina MiSeq sequencer. Sequences were assembled using Velvet, annotated using Prokka, and mapped to the published TIGR4 and D39 (R000000036) reference genomes. Bases and single-nucleotide variants were identified using the SAMtools “mpileup” command and IGVTools. Sites were filtered to a minimum depth of five reads at each and single-nucleotide variant quality of 25, and the Integrated Genome Viewer was used to visualize mapping and coverage. S. pneumoniae was cultured overnight at 37°C in 5% CO₂ on Columbia agar (Oxoid) supplemented with 5% horse blood (TCS Biosciences). Working stocks grown to an OD of 0.4 (\(10^9\) CFU/ml) were made using Todd-Hewitt broth supplemented with 0.5% yeast extract and stored at −80°C in 10% glycerol as single-use aliquots. CFU were confirmed by colony counting of \(\log_{10}\) serial dilutions of bacteria cultured overnight on 5% Columbia blood agar. Chloramphenicol (10 \(\mu\)g/ml) and kanamycin (500 \(\mu\)g/ml) were added to blood agar plates where appropriate.

**Preparation of bacterial lysates and Triton X-114 extraction of lipoproteins**

Bacterial lysates were made using mid log-phase growth S. pneumoniae cells by addition of 0.1% deoxycholate (Sigma-Aldrich) in PBS for 30 min at 37°C and sonication with a Soniprep 150 (Sanyo) ultrasonicator. Membrane-associated proteins were extracted from lysates by Triton X-114 extraction, as described previously (48, 49), washed, and diluted 1:2 in PBS prior to solubilization in Laemmli sample buffer for SDS-PAGE and visualization using Coomassie brilliant blue (Sigma-Aldrich) staining.

**Cell isolation and culture**

Blood samples were obtained from healthy volunteers or IRAK-4–deficient subjects homozygous for the Q259X mutation for isolation of PBMC or production of monocye-derived macrophage (MDM) by differentiation with M-CSF, as previously described (50). Bone marrow was extracted from 6- to 8-week-old C57BL/6 WT, TLR2−/−, TLR4−/− (Jackson ImmunoResearch Laboratories), or Myd88<trif/> mice (gift of S. Akira, Department of Host Defense, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka, Japan) and differentiated into bone marrow–derived macrophages (BMDM) for 7 d in L929-conditioned medium using standard protocols (51, 52). The RAW 264.7 murine macrophage cell line was cultured as adherent cells in RPMI 1640 (Life Technologies) supplemented with 2 mM L-glutamine (Invitrogen) and 10% FBS (Life Technologies).

**PGN purification and structural analysis**

Pneumococcal cell wall (PGN–teichoic acid complex) and PGN were purified, as described (53), and muropeptide was released by digesting PGN with cellosyl (provided by Hoechst). Muropeptides were reduced with sodium borohydride and analyzed by high-pressure liquid chromatography, as described (53). The peaks were assigned by comparing their retention time with the retention time of known muropeptides obtained from strain R6, whose muropeptide profile is similar to that of strain TIGR4. Peak 1 (Fig. 3) was collected and analyzed by electrospray mass spectrometry, as described (53, 54).

**Innate immune stimulation, cytokine measurements, and NF-κB nuclear translocation**

HEK TLR2 reporter assays were stimulated with S. pneumoniae for 16 h, according to the manufacturer’s instructions (Invivogen). Whole cells were stimulated as follows for collection of supernatants, RNA isolation, or NF-κB translocation assays: MDM and PBMC stimulated for 1–24 h with S. pneumoniae strains at a multiplicity of infection (MOI) of 5–50 or Pam3CSK4 (100 ng/ml; Axis-Shield); RAW 264.7 cells stimulated with different S. pneumoniae strains at a MOI of 5 or 10 μl (in a total volume of 300 μl) Triton X-114 extracts for up to 4 h (47); BMDM stimulated with S. pneumoniae bacterial strains in DMEM without any supplements for 4 h at a MOI of 5. Cytokine levels were measured in cell culture supernatants using ELISA (R&D Systems or eBioscience), or the Luminex or MSD platforms, according to the manufacturer’s instructions. NF-κB activation was assessed by quantifying nuclear RelA by using the MSD system, according to the manufacturer’s instructions, or using immunofluorescence of MDMs to obtain nuclear/cytoplasmic ratios of NF-κB Rel A (p65) staining as a marker of NF-κB nuclear translocation (50, 55).

**Transcriptional profiling by cDNA microarray and quantitative PCR**

Total RNA was purified from cell lysates using the RNeasy mini kit (Qiagen). Samples were processed for Agilent microarrays, and data were normalized, as previously described (56). Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-1541. For quantitative PCR (qPCR), cDNA was synthesized using the qScript cDNA Supermix kit (Quanta Biosciences), and qPCR of selected genes was performed using the following primers. (Applied Biosystems): IL23 (Hs00372234_m1), IL18 (Hs01555410_m1), IL6 (Hs00985639_m1), and TNFa (Hs00171428_m1). PTG2 expression was quantified using the following: forward primer, 5′-CCGGTGACTAAATCCAGCAATATCGT-3′; reverse primer, 5′-CCGGTGACTAAATCCAGCAATATCGT-3′;
and probe, 5′-CCATACGCAAATCCCTT-3′ (Applied Biosystems). Expression levels of target genes were normalized to GAPDH, as previously described (57).

Animal model of lung infection

Six- to 8-wk-old outbred CD1 female white mice (Charles Rivers Breeders) were inoculated intranasally with 1 × 10⁷ CFU in an inoculum volume of 50 μl under halothane (Zeneca) general anesthesia. Bronchoalveolar lavage fluid (BALF) samples were obtained from culled mice 4 h postinfection for cytokine assays using ELISAs (R&D Systems), bacterial CFU by colony counting of serial dilutions plated onto Columbia blood agar plates containing 5 μg ml⁻¹ gentamicin, and total cell counts using a manual hemocytometer (Gova Glasstic slides; Hycor Biomedical UK) under light microscopy (original magnification ×20) following 1:2 dilution of BALF in 0.001% crystal violet in acetic acid.

Statistical analysis

Data that would be normally distributed (results of macrophage cytokine analyses and qPCR) were analyzed using paired (for data obtained from matched samples) or unpaired (for data obtained from unmatched samples) t tests. Nonnormally distributed data (data from mouse experiments and NF-κB nuclear translocation data) were compared using the Mann–Whitney U test. Principal component (PC) analysis was used to compare global gene expression profiles, as previously described, and t tests were used to identify significant gene expression differences (p < 0.05) between samples using MultiExperiment Viewer v4.6.0 (57). Transcriptional regulation of specific gene signatures was assessed by analysis of single transcription factor binding site enrichment analysis (http://opossum.cisreg.ca/oPOSSUM3). Pathway and Gene Ontology overrepresentation analysis was performed using InnateDB (http://www.innatedb.com/).

Results

TLR2 signaling in response to S. pneumoniae is dependent on lipoproteins

Triton-X extracts of the TIGR4 Δlgt S. pneumoniae strain confirmed that this strain has almost no detectable lipoproteins, similar to the previously described 0100993 Δlgt strain (Fig. 1A) (45). A reporter assay was used to assess whether loss of lipoproteins significantly affected TLR2 signaling by S. pneumoniae (Fig. 1B). Live whole TIGR4 WT bacteria stimulated a strong TLR2-dependent signal, whereas live Δlgt bacteria did not cause any significant response even with high multiplicities of infection (MOI). Lysed bacteria gave similar results, demonstrating that impaired growth or failure of release of nonlipoprotein TLR2 ligands such as PG and LTA was not responsible for the lack of

FIGURE 2. Macrophage TNF-α responses are dependent on S. pneumoniae lipoproteins. (A and B) Time course of TNF-α concentrations (measured by ELISA) in RAW cell culture supernatants after incubation with TIGR4 or TIGR4 Δlgt S. pneumoniae (MOI 5). (C) TNF-α concentrations in RAW cell culture supernatants after 4-h incubation with Pam3CSK4, WT, TIGR4 Δlgt, TIGR4 ΔpabB, or TIGR4 Δlgt ΔpabB S. pneumoniae. Bacterial CFU after 4-h incubation are stated above each column. (D) TNF-α concentrations in RAW cell culture supernatants after incubation for 4 and 24 h with the TLR agonist Pam3CSK4, or sonicated TIGR4 or TIGR4 Δlgt S. pneumoniae. (E) TNF-α concentrations in RAW cell culture supernatants after 4-h incubation with TIGR4 or TIGR4 Δlgt S. pneumoniae or their corresponding 3.3% Triton X-114 lipoprotein. For all panels, n = 3–4 and is representative of repeated experiments, data are presented as means, error bars represent SEMs, and p values were obtained using unpaired t tests.
TLR2 response to the Δlgt mutation. We have not been able to complement the Δlgt mutation (45); hence, to confirm linkage of the loss of TLR2 responses to the mutation, we transferred the mutation to the D39 S. pneumoniae strain and obtained similar results to the TIGR4 strain, with almost complete loss of TLR2-dependent signaling to both live and lysed D39Δlgt (Fig. 1C). Whole-genome sequencing of the Δlgt TIGR4 and D39 S. pneumoniae strains was performed to identify unexpected mutations that might confound the results (Supplemental Table I). Both the D39 and TIGR4 Δlgt strains contained the expected complete deletion of lgt (replaced by the kan antibiotic resistance gene), as well as two synonymous single nucleotide polymorphisms (SNPs) in the gene immediately downstream of lgt (Spd_1244 and Sp_1413, respectively) that encodes a Hpr(Ser) kinase/phosphatase. In addition, the Δlgt TIGR4 strain contained one SNP in a noncoding intergenic region, and one nonsynonymous SNP changing a histidine for an arginine at position 336 of a 413-aa residue protein encoded by Sp_2175 (dltB), predicted to be an alanine exporter involved in lipoteichoic acid synthesis. These data indicate that loss of TLR2 stimulation was linked to deletion of lgt and suggest that lipoproteins are major S. pneumoniae TLR2 ligands.

Mouse macrophage TNF-α response to S. pneumoniae is dependent on lipoproteins

To assess the significance of lipoprotein-dependent TLR2 signaling on the strength of the inflammatory response to S. pneumoniae, TNF-α secretion by the RAW mouse macrophage cell line was compared after incubation with WT or Δlgt TIGR4. Significant production of TNF-α was evident after 2-h incubation with WT S. pneumoniae (Fig. 2A), but was significantly attenuated in response to the Δlgt strain at 2, 4, and 24 h (Fig. 2A, 2B). Loss of lipoproteins affects S. pneumoniae growth (45), and after 4-h incubation with RAW cells there were ~60% fewer Δlgt bacteria/ml than the WT strain (Fig. 2C). However, the reduced TNF-α response to the Δlgt strain persisted when RAW cells were stimulated with sonicated bacteria (Fig. 2D) and after transfer of the lgt mutation to the ΔspaΔbb strain (Fig. 2C), an auxotrophic mutant that replicates poorly without addition of exogenous paraaminobenzoic acid (47). Furthermore, there was a similar scale reduction in the RAW cell TNF-α response to the Δlgt strain when RAW cells were stimulated with Triton-X extracts for 4 h, providing further evidence of a direct effect of lipoproteins on inflammatory responses (Fig. 2E).

The Δlgt mutation has no effect on PGN structure

As the PGN synthesis enzyme PGN deacetylase is a hypothetical lipoprotein, PGN structure could potentially be affected by deletion of lgt (58). However, the muropeptide profile of the Δlgt TIGR4 strain did not significantly differ from that of WT TIGR4, and both were similar to the reported muropeptide profile of strain R6 (an unencapsulated derivative of D39) (53). Importantly, PGN from Δlgt TIGR4 contained peaks corresponding to muropeptide with deacetylated GlcNac residues, which are generated by PGN deacetylase (53, 58). To further confirm the presence of deacytlylated muropeptides in the Δlgt TIGR4 sample, the main deacetylated monomer (peak 1, Fig. 3) was analyzed by mass spectrometry (MS). The obtained neutral mass of 783.3880 Da is consistent with the theoretical mass of 783.3862 Da for the deacetylated disaccharide tripeptide (GlcN-MurNac-L-Ala-D-iGln-L-Lys). The MS/MS fragmentation pattern of this signal showed the expected loss of a dehydrated glucosamine residue (160.9152 Da, theoretical 161.0688). Therefore, there are no significant alterations in PGN structure that could account for the effects of the Δlgt mutation on inflammatory responses to S. pneumoniae.


**FIGURE 4.** S. pneumoniae lipoproteins induce proinflammatory cytokines via TLR2 pathway activation. Mean TNF-α concentrations in cell culture supernatants from BMDMs obtained from C57BL/6 mice incubated for 4 h with the TLR agonists (LPS and/or Pam3CSK3), or TIGR4 or TIGR4Δlgt S. pneumoniae. (A) Results for BMDMs obtained from WT or Myd88−/− or TLR2−/− mice. (B) Results for BMDMs obtained from WT or TLR2−/− mice. (C) Results for BMDMs obtained from WT or TLR2−/− mice. For all panels, n = 3–4 and is representative of repeated experiments, error bars represent SEMs, and p values were obtained using unpaired t tests.
S. pneumoniae lipoprotein effects on macrophage TNF-α responses are TLR2 dependent

To investigate whether the differences in TNF-α responses between the WT and Δlgt strains were attributable to specific TLRs, TNF-α responses by BMDMs from WT, tlr2−/−, tlr4−/−, or Myd88/trif−/− mice were also evaluated. There was no significant TNF-α release by BMDMs from Myd88/trif−/− mice with WT (TIGR4) or lipoprotein-deficient (Δlgt) S. pneumoniae (Fig. 4A). WT TIGR4 induced significantly greater TNF-α secretion by BMDMs from WT mice than the Δlgt strain. TNF-α release by BMDMs from tlr2−/− mice was strongly reduced in comparison with BMDMs from WT mice, although there was still a residual response compared with BMDMs from WT mice, indicating that the increase seen with BMDMs from WT mice was TLR2 dependent. In contrast, although TNF-α production by BMDMs from tlr4−/− mice was reduced in response to both strains compared with BMDMs from WT mice, WT TIGR4 still induced significantly higher levels of TNF-α secretion than the Δlgt strain (Fig. 4C). These results demonstrate that the TLR2 but not the TLR4 response was dependent on bacterial lipoproteins.

Loss of lipoproteins attenuates NF-κB activation in response to S. pneumoniae

We then assessed whether loss of TLR2 signaling in response to the Δlgt strain affected activation of the key proinflammatory transcriptional regulator NF-κB (59, 60) in macrophages. Quantifying NF-κB Rel A in nuclear extracts showed that the increase in BMDM NF-κB activation by WT TIGR4 that was largely abrogated in macrophages from Myd88/trif−/− mice or in WT BMDMs infected with the Δlgt strain (Fig. 5A). Similarly, NF-κB activation was significantly attenuated in response to the Δlgt strain compared with WT TIGR4 when assessed using a confocal assay to quantify nuclear translocation of NF-κB Rel A in human MDMs (Fig. 5B, 5C). These data suggest both mouse and human macrophage NF-κB activation in response to S. pneumoniae is dependent on TLR recognition of lipoproteins.

Comparison of the macrophage transcriptome to the TIGR4 and Δlgt strains

To provide detailed data on the effect of lipoproteins on global inflammatory responses to S. pneumoniae, human MDM genome-wide transcriptional responses were compared after incubation for 4 h with live WT or Δlgt TIGR4 bacteria, or the specific TLR2 agonist Pam2CSK4. All three stimuli caused major changes in gene expression; Pam2CSK4 and WT TIGR4 stimulated the increased expression of 854 and 936 genes, respectively, with the Δlgt strain causing upregulated expression of slightly fewer genes (591 in total) (Fig. 6A, 6B). Although there was a large overlap in the genes upregulated by Pam2CSK4, WT TIGR4, and the Δlgt strain, there was greater overlap with Pam2CSK4 for responses to WT TIGR4 than for Δlgt, in keeping with the hypothesis that S. pneumoniae lipoproteins are important for TLR2-associated transcriptional responses.

PC analysis was used to explore differences in cocorrelated gene expression data. In PC1, which reflects the greatest gene expression differences between stimulated and unstimulated macrophages, the effects of WT TIGR4 were equivalent to those of Pam2CSK4. However, Δlgt-induced changes in gene expression were reduced for this component (Fig. 6C, 6D). In contrast, the Δlgt and WT TIGR4 strains induced comparable changes to gene expression in PC2, distinct from those induced by Pam2CSK4 and therefore by

FIGURE 5. Pneumococcal lipoproteins are important determinants of TLR-induced NF-κB activation. (A) Quantification of nuclear NF-κB p65 using MSD (arbitrary units) for BMDMs from C57BL/6 WT and Myd88/trif−/− mice incubated for 1 h with Δlgt and TIGR4 S. pneumoniae (MOI of 5). Each symbol represents results for one well, the bar represents median values, and the p value for comparison of TIGR4 and Δlgt strains incubated with WT BMDMs was obtained using a Mann–Whitney U test. (B) Confocal immunofluorescence images of NF-κB Rel A in human MDM from five different donors stimulated for 2 h with WT (TIGR4) or lipoprotein-deficient (Δlgt) S. pneumoniae (MOI 5 or 50) showing diminished nuclear translocation of RelA (pink nuclei) for cells incubated with the Δlgt strain. (C) Quantitative image analysis of the median (IQR) ratio of nuclear to cytoplasmic RelA staining (*p = 0.0079, Mann–Whitney U test).
inference independent of TLR2 (Fig. 6C, 6E). This analysis is consistent with the Venn diagram of qualitative transcriptional responses to these stimuli, which showed that MDMs stimulated with the \( \Delta \)lgt and TIGR4 strains shared increased expression of 320 genes that were not upregulated following specific TLR2 stimulation by Pam2CSK4 (Fig. 6B). Bioinformatic analysis of the top 50 genes that reflected \( S. \) pneumoniae TLR2-dependent responses in PC1 and TLR-2 independent responses in PC2 is presented in Supplemental Fig. 1. Transcription factor binding site enrichment analyses revealed the dominance of NF-\( \kappa \)B–regulated genes in PC1, associated with enrichment for proinflammatory cytokines reflected by pathway and gene ontology analysis (Supplemental Fig. 1). In contrast, TLR2-independent responses to \( S. \) pneumoniae reflected in PC2 showed markedly less enrichment for NF-\( \kappa \)B–regulated genes, with modest enrichment for genes associated with nucleosome and transcriptional regulation instead. Taken together, these data suggest that the canonical inflammatory responses to TIGR4 are mediated by TLR2 and are attenuated in response to the \( \Delta \)lgt strain. However, non-TLR2 transcriptional responses were largely comparable in MDMs stimulated with either the \( \Delta \)lgt or TIGR4 strains.

The genes showing the greatest differences in expression between MDMs stimulated with \( \Delta \)lgt and TIGR4 strains included the cytokines IL-23, IL-6, and IL-1\( \beta \), and the chemokines CXCL1, CXCL2, and CXCL3 (Supplemental Fig. 2A), and transcriptional factor binding site analysis showed enrichment for regulation by NF-\( \kappa \)B (Table I) in keeping with the data shown in Fig. 5 and Supplemental Fig. 1. Selected differences in expression of prototypic inflammatory genes identified by microarray were validated by qPCR. IL-23, IL-6, and PTGS2 expression were all significantly lower in macrophages stimulated with the \( \Delta \)lgt strain compared with WT TIGR4, confirming the transcriptome results.
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Table I. Transcription factor enrichment of gene expression differences between MDMs stimulated with TIGR4 and Δlgt

| Transcription Factor | Background TFBS Rate | Target TFBS Rate | Z-Score* | p
|----------------------|----------------------|-----------------|---------|---
| RELA                 | 0.0035               | 0.0103          | 26.16   | NS
| NF-κB                | 0.0050               | 0.0118          | 22.11   | NS
| REL                  | 0.0081               | 0.0167          | 21.66   | NS
| HLF                  | 0.0049               | 0.0109          | 19.48   | NS
| NFκB1                | 0.0020               | 0.0053          | 17.10   | NS
| NFIL3                | 0.0033               | 0.0066          | 13.19   | NS
| STAT1                | 0.0016               | 0.0038          | 12.10   | NS
| Foxq1                | 0.0060               | 0.0100          | 11.71   | NS
| Ar                   | 0.0006               | 0.0017          | 10.32   | NS

*Z-scores of >10 are considered to indicate highly significant overrepresentation of TFBS within the analyzed gene list.

Table II. Mean (SEM) cytokine responses in pg/ml 4 h after incubation of PBMCs from five donors with the TIGR4 wild-type and Δlgt S. pneumoniae strains

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Conditions</th>
<th>TIGR4</th>
<th>Δlgt</th>
<th>p for TIGR4 vs. Δlgt</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>Unstimulated</td>
<td>34 (9.6)</td>
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<td>56 (16)</td>
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<td>IL-10</td>
<td>Pam3CSK4</td>
<td>4.7 (1.2)</td>
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<td>IL-12</td>
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<td>3.1 (1.3)</td>
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<td>10 (1.1)</td>
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<td>IL-1β</td>
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<td>Pam3CSK4</td>
<td>127 (25)</td>
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<td>245 (57)</td>
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<td>TNF-α</td>
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<td>690 (202)</td>
<td>8,030 (2,160)</td>
<td>4,710 (965)</td>
</tr>
</tbody>
</table>

The p values are for comparisons of results obtained with the TIGR4 or Δlgt strains (unpaired t tests).

Discussion

S. pneumoniae infections cause a strong NF-κB–mediated proinflammatory response that is essential for host immunity but also causes some of the important features of severe infection (2–4, 60). This inflammatory response has previously been shown to be partially dependent on TLR2 (18, 19, 23, 24, 61). In addition, TLR2 responses are required for Th17 and some humoral adaptive immune responses (19, 28, 29). Identifying the S. pneumoniae TLR2 ligands is important for our understanding of disease pathogenesis and for the design of therapeutic manipulations aimed at modifying inflammatory responses or improving humoral and cellular immunity to S. pneumoniae. Although the cell wall components PGN and LTA are proinflammatory S. pneumoniae ligands thought to be recognized by TLR2 (33–35), recent evidence suggests they may stimulate inflammatory responses by TLR2-independent mechanisms (36–38). In contrast to the extensive data on PGN and LTA, the potential role of lipoproteins as proinflammatory stimuli during S. pneumoniae infections has not been characterized. We have now explored the role of lipoproteins as S. pneumoniae proinflammatory ligands using the Δlgt strain (45), which allows the effects of lipoproteins to be assessed in the context of the whole bacterium rather than relying on purified products.

Our results demonstrated that S. pneumoniae lipoproteins are major contributors to the macrophage TLR- and NF-κB–mediated inflammatory response. In theory, the effects of the Δlgt mutation on the physiology of S. pneumoniae (45) could have confounded these results, but control experiments demonstrated persistent...
decreases in macrophage inflammatory responses to the Δlgt strain when live or nonreplicating (to control for bacterial CFU) bacteria were used. The data obtained by the nonreplicating ΔpabB strains could also have been confounded by the dual mutation in the Δlgt/pabB strain compared with the ΔpabB strain; however, additional control experiments also showed reduced inflammatory responses to lipoprotein extracts or sonicated bacteria (to release cell wall fragments) from the Δlgt strain compared with WT. Differences in TNF-α responses to TIGR4 and Δlgt strains were lost for macrophages from TLR2 mice and preserved for macrophages from TLR4 mice. Of the surface structures that affect interactions with the host, S. pneumoniae LTA has recently been shown not to affect macrophage proinflammatory gene transcriptional responses to the Δlgt strain. This suggests that lipoproteins may also play a role in the inflammatory response to S. pneumoniae, as these are major TLR2 ligands for S. pneumoniae. LTA is not affected by deletion of the capsule polysaccharide or lipoprotein. Hence, it is unlikely that the differences in inflammatory responses seen between WT and Δlgt strains were confounded by effects of the mutation on bacterial physiology, PGN structure, or the capsule. Complementing the Δlgt mutation would have been beneficial, but for poorly understood reasons S. pneumoniae mutant strains can be difficult to complement, and our attempts to complement the Δlgt mutant failed (45). Instead, we used whole-genome sequencing and transfer of the mutation to another strain to link the phenotypes seen to deletion of lgt. These results indicate that lipoproteins are major TLR2 ligands for S. pneumoniae, similar to results obtained with other Gram-positive pathogens (39, 40, 51, 65).

We used transcriptional arrays, qPCR, and supernatant cytokine levels to confirm decreased expression of a variety of proinflammatory cytokines and chemokines for macrophages infected with the Δlgt strain. There were some discrepancies between transcriptional and protein level data with, for example, consistently reduced levels of TNF-α protein in response to infection of MDMs, BMDMs, and PBMCs despite little change in gene transcription at the 4-h time point, which may reflect release of pre-formed TNF-α. In addition, significant levels of IL-1β, IL-6, and TNF-α were still produced by leukocytes in response to the Δlgt strain, potentially reflecting inflammatory responses induced by NOD2-mediated recognition of PGN and pneumolysin activation of the inflammasome and/or TLR4 (16, 27, 36, 37, 66, 67). These mechanisms combined with residual TLR2 activation may explain why the macrophage proinflammatory gene transcriptional responses to the Δlgt strain were reduced rather than absent compared with WT TIGR4. These results are also consistent with published data showing more marked effects of MyD88 deficiency than loss of individual TLRs on inflammatory response to S. pneumoniae in mice and humans (8, 11, 17, 19, 21). As TLR2 activation increases TLR4-, pneumolysin-, and NOD2-dependent inflammatory responses to S. pneumoniae (21, 26, 27), S. pneumoniae lipoproteins may also increase inflammatory responses indirectly through these other mechanisms of innate immune recognition.

The global assessment of macrophage responses by transcriptional profiling significantly improves our understanding of key interactions of the host with S. pneumoniae. S. pneumoniae caused major changes in macrophage gene expression, with upregulation of >900 genes in response to the WT TIGR4 strain. Many genes showing large differences in expression between control macrophages and those infected with TIGR4 S. pneumoniae were equally expressed in response to infection with the Δlgt strain. However, the small proportion of genes with differences in expression between the WT and Δlgt strain was concentrated in the transcriptional responses with the greatest upregulation in response to S. pneumoniae (PC1), which also responded to Pam3CSK4 stimulation. These data show that macrophage transcriptional responses to S. pneumoniae are dominated by TLR2-dependent genes that often require the presence of lipoproteins for maximal stimulation, with a larger number of generally weaker changes in expression of genes whose responses are TLR2 independent. The list of TLR2-dependent genes has a striking preponderance for genes encoding important proinflammatory proteins that bioinformatic analysis suggests are largely activated by the NF-κB pathway, in keeping with the data showing reduced
NF-κB activation in response to the Δlgt strain. Our data contrast with those showing that pneumolysin induced macrophage expression of very few genes encoding chemokines or cytokines (68).

The potential clinical relevance of S. pneumoniae lipoprotein-dependent inflammation was assessed in a mouse model of pneumonia, and using PBMCs from individuals with IRAK-4 deficiency. We were able to demonstrate that the rapid TNF-α and IL-1β responses during early lung infection with S. pneumoniae were reduced in response to the Δlgt strain. Despite the effect of lipoproteins on the IL-6 response in vitro, BALF IL-6 responses were similar for mice infected with the TIGR4 or Δlgt strain. Potentially, differences in IL-6 levels could develop later in infection, but as the Δlgt strain cannot maintain long-term infection in mouse models (45), it was not feasible to investigate this.

The striking susceptibility of children with IRAK-4 deficiency to invasive S. pneumoniae infections demonstrates the vital importance of inflammatory responses for immunity to this pathogen during childhood (5, 6, 9, 10), but the expressivity of these cytokines by healthy controls was largely dependent on lipoproteins. Importantly, WT PBMCs IL-1β, IL-6, and TNF-α responses to the Δlgt strain were attenuated at the transcriptional and (for IL-1β and TNF-α) protein level, similar to the results for WT TIGR4 incubated with PBMCs obtained from IRAK4-deficient subjects. Due to the limited number of donors available, only two IRAK4-deficient subjects were available for testing. Despite this, the results were highly consistent between the two donors and suggest that lipoproteins are important ligands driving IRAK-4-dependent inflammatory responses and therefore protective immunity in children.

Overall, our data demonstrate that lipoproteins are major S. pneumoniae TLR2 ligands that are required for the maximum transcriptional response for many of the dominant macrophage gene responses to S. pneumoniae, including induction of IRAK-4-dependent protective cytokines. Specific targeting of bacterial lipoprotein/TLR2 inflammatory responses could be a novel therapeutic approach for enhancing cellular and humoral immune responses to vaccines or, when combined with effective antibiotic therapy, for improving the outcome of severe S. pneumoniae infections.

Acknowledgments

We thank Joe Gray (Pinnacle Proteomics Facility, Institute for Cell and Molecular Biosciences, Newcastle University) for mass spectrometry analysis.

Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Table 1.

Single nucleotide polymorphisms identified in the genome sequences of the TIGR4 and D39 Δlgt strains compared to their parental strains.

<table>
<thead>
<tr>
<th>Genome position</th>
<th>Gene affected</th>
<th>Reference bp / codon</th>
<th>Mutant bp / codon</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D39Δlgt</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>1272512</td>
<td>SPD_1244 hprK</td>
<td>ATC</td>
<td>ATA</td>
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<tr>
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<td>SPD_1244 hprK</td>
<td>GCG</td>
<td>GCT</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>730678</td>
<td>Intergenic region</td>
<td>C</td>
<td>T</td>
<td>n/a</td>
</tr>
<tr>
<td>1332449</td>
<td>SP_1413 hprK</td>
<td>GCG</td>
<td>GCT</td>
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<tr>
<td>1332584</td>
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<td>AAT</td>
<td>AAC</td>
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<tr>
<td>2092182</td>
<td>SP_2175 dltB</td>
<td>CAT</td>
<td>CGT</td>
<td>Non-synonymous mutation: changes a histidine for an arginine at position 336 of 413</td>
</tr>
</tbody>
</table>
The top 50 transcriptional responses associated with principal component (PC)1 and PC2 in Figure 6D-E were subjected to transcription factor binding site (TFBS) enrichment analysis using oPossum (http://opossum.cisreg.ca/oPOSSUM3/), and to Gene Ontology and Pathway enrichment analyses using innateDB (http://www.innatedb.com/). In each case the test statistic (Z-score or corrected P value) and the number of genes associated with each annotation is presented as an individual data point. A Z-score >10 and −Log_{10} P>1.3 are considered statistically significant.
(A) Gene expression heat map for mean relative gene expression levels and fold differences for the 42 genes showing significant differences in expression ($P < 0.05$) between human monocyte derived macrophages (MDM) from at least three different donors stimulated for four hours with TIGR4 or Δlgt S. pneumoniae. (B-F) Validation using qPCR (normalised to GADPH expression) of selected pro-inflammatory genes showing reduced expression in the transcriptional array analysis after incubation with Δlgt strain compared to TIGR4. (G) Relative supernatant levels of IL-6 and TNFα (measured using a luminex system) in cell culture supernatants of MDMs incubated with the Δlgt strain expressed as a percentage of the result for the same donor when incubated with the TIGR4 strain. For panels (B) to (G) each symbol represents results from a single donor, and the bars medians. $P$ values are for comparisons of results for MDM stimulated with the TIGR4 or Δlgt strains (paired t tests).
IL-6 concentrations in cell culture supernatants from BMDMs obtained from C57BL/6 and TLR2-/- mice incubated for 4 hours with the TLR2 agonist Pam2CSK4, or TIGR4 or TIGR4Δlgt S. pneumoniae strains. n = 3 to 4, error bars represent SEMs, and the stated P values were obtained using unpaired t tests.