Analysis of the i*n vitro* transcriptional response of human pharyngeal epithelial cells to adherent *Streptococcus pneumonia*: evidence for a distinct response to encapsulated strains

Running title: Transcriptional response to pneumococcal adherence

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ABSTRACT **NB: 250 words or fewer!!**

Infection of the human host by *Streptococcus pneumoniae* begins with colonization of the nasopharynx, which is mediated by adherence of bacteria to respiratory epithelium. Several studies have indicated an important role for the pneumococcal capsule in this process. Here, we used microarrays to characterize the *in vitro* transcriptional response of human nasopharyngeal epithelial Detroit 562 cells to adherence of serotype 2-encapsulated strain D39, serotype 19F-encapsulated strain G54, serotype 4-encapsulated strain TIGR4, and their nonencapsulated derivatives (Δ*cps*). In total, 322 genes were found to be upregulated in response to adherent pneumococci. Twenty-two genes were commonly induced, including those encoding several cytokines (e.g., IL-1β, IL-6), chemokines (e.g., IL-8, CXCL1/2), and transcriptional regulators (e.g., FOS), consistent with an innate immune response mediated by Toll-like receptor signaling. Interestingly, 85% of genes were induced specifically by one or more encapsulated strains, suggestive of a capsule-dependent response, although purified type 2 polysaccharides alone had no effect. Over a third of these loci encoded products predicted to be involved in transcriptional regulation and signal transduction, in particular the MAPK signaling pathways. Real-time PCR of a subset of ten genes confirmed microarray data and showed a time-dependent upregulation of especially innate immunity genes. Downregulation of epithelial genes was most pronounced upon adherent D39Δ*cps*, as 68% of the 161 genes identified was only repressed using this nonencapsulated strain. In conclusion, we identified a subset of host genes specifically induced by encapsulated strains during *in vitro* adherence, underlining the complexity of interactions occurring during the initial stages of pneumococcal infection.

INTRODUCTION

The gram-positive bacterium *Streptococcus pneumoniae* is a major cause of mucosal infections such as otitis media, and of more severe invasive diseases like pneumonia and septicemia. Particularly children, elderly people, and immunocompromized patients are affected by this pathogen. Colonization of the nasopharynx, mediated by adherence of the bacterium to epithelial cells of the upper respiratory tract, is common and usually asymptomatic. It is, however, also the critical first step for infection of the host by *S. pneumonia*e (7). In addition, nasopharyngeal carriage of *S. pneumoniae* has been proposed to serve as a main source for transmission of this pathogen within the community (16). Neither host nor bacterial factors required for adherence and colonization have been completely characterized yet.

S. pneumoniae expresses several proteins described to be important for its virulence, such as pneumolysin and choline-binding proteins (CBPs) (reviewed in (35)). Of these, CbpA (also known as PspC) and CbpG have been shown to function as an adhesin for eukaryotic cells (34,45). Another major virulence factor is the polysaccharide capsule, of which at least 90 different serotypes are known (19). Pneumococci can occur in different colony variants known as opaque and transparent phase variants, characterized by high and low levels of capsule production, respectively. Generally, transparent phase variants display enhanced adherence and colonization ability compared to opaque variants (24,53). Apparently, reduced capsule expression (down to 20% of wild-type levels) benefits the initial adherence and colonization of pneumococci, possibly by allowing better exposure of surface proteins important for adherence (32). In line with this, pneumococci in close contact with murine or cultured epithelial cells appeared to contain substantially lower levels of capsule compared to bacteria more distant from cells (17). Interestingly, a recent study showed that, despite its

72 inhibitory effect on adherence, the presence of capsule does confer an advantage during
73 colonization: only encapsulated strains are able to transit efficiently from the luminal mucus
74 to the epithelial surface (40). Finally, there is an absolute requirement for capsule in systemic
75 infections, with varying degrees of severity depending on capsular serotype (9,49). Once
76 inside the host, the capsule is considered to protect *S. pneumoniae* against host immunity,
77 preventing, for instance, complement-mediated opsonophagocytosis (1).

78 The innate immune system provides a critical non-specific first line of defense against
79 microorganisms, and is essential for proper development of subsequent adaptive immune
80 responses. Key elements of innate immunity are the so-called pattern recognition receptors
81 (PRRs), of which the membrane-bound Toll-like receptors (TLRs) play a central role in the
82 response to microbes (22). TLRs recognize conserved microbial structures referred to as
83 pathogen-associated molecular patterns, such as those occurring in bacterial lipoteichoic acid
84 (LTA), lipopolysaccharide (LPS), and DNA (unmethylated CpG motifs). Involvement of
85 both TLR2 and TLR4 in the host response to pneumococci has been demonstrated using
86 various *in vitro* and *in vivo* infection models, mediated by recognition of pneumococcal LTA
87 (TLR2) and pneumolysin (TLR4) (18,33,48,50,52). Recent studies also indicated a role for
88 TLR9 in host defense against pneumococcal infection, particularly at the early stages (2,36).
89 The central signaling adaptor myeloid differentiation factor-88 (MyD88) mediates signal
90 transduction from most of the TLRs upon ligand binding (22), and has been shown to be
91 crucial for the host response to pneumococcal infection (3,9,23,26). Subsequent activation of
92 the nuclear factor-κB (NF-κB) pathway leads to production of proinflammatory cytokines
93 such as TNFα, IL-1, and IL-6.

94 DNA microarray technology is particularly suitable to examine the host response on a
95 global scale, as several thousand genes can be surveyed at once. Thus far, only a few studies
96 have taken this approach to identify host factors involved in pneumococcal colonization and

subsequent disease. Rogers *et al.* have analyzed the transcriptional response of a human monocytic cell line to pneumococci, focusing on pneumolysin-dependent and -independent gene expression (44). They identified 40 genes responsive independent of pneumolysin, and 142 pneumolysin-responsive genes, including those encoding IL-8, MIP-1β, lysozyme, and caspases 4 and 6 (44). Examination of gene expression profiles in middle ears of rats during early and late stages of *S. pneumoniae*-induced otitis media identified transient expression of cytokines and persistent upregulation of several transcription factors including c-jun and c-myc (11,30). Similarly, transcriptional profiling of cortex and hippocampus of rats during acute pneumococcal meningitis identified 598 differentially regulated genes, associated mainly with growth control, signal transduction, cell death/survival, cytoskeleton, and innate and adaptive immunity (12). Finally, a microarray-based screen for host genes with increased expression during colonization of the murine nasal mucosa identified siderocalin, a component of innate immunity involved in iron-sequestering (39).

As mentioned, the first interaction between the pneumococcus and the host in colonization and subsequent infection occurs during adherence of the bacterium to the respiratory epithelial cell surface. To gain molecular insight into the response of the epithelium during this interaction, we used microarrays to characterize the transcriptional profile of the human nasopharyngeal epithelial cell line Detroit 562 after 2 hours adherence of *S. pneumoniae* wild-type strains D39 (serotype 2), G54 (serotype 19F), and TIGR4 (serotype 4). Considering the crucial role of pneumococcal capsule in this process, we simultaneously analyzed the response to the three corresponding isogenic capsule-locus deletion mutants (D39Δ*cps*, G54Δ*cps*, and TIGR4Δ*cps*), as well as to purified type 2 capsular polysaccharides. This approach allowed us to identify epithelial genes responsive in a capsule-dependent and -independent fashion. Finally, we used real-time PCR to follow expression of a small selection of genes over time.

MATERIALS AND METHODS

Cell culture. The human pharyngeal epithelial cell line Detroit 562 (ATCC CCL-138) was grown and maintained at 37°C in a 5% CO_2 atmosphere in RPMI 1640 without phenol red (Invitrogen, The Netherlands) supplemented with 1 mM sodium pyruvate and 10% (vol/vol) fetal calf serum (FCS).

Pneumococcal strains, growth conditions, and pneumococcal polysaccharides. *S. pneumoniae* strains used in this study were the wild-type isolates D39 (D39WT; serotype 2; NCTC 7466), G54 (G54WT; serotype 19F; (13)), TIGR4 (51) and their isogenic capsule-locus (*cps*) deletion mutants D39Δ*cps*, G54Δ*cps*, and TIGR4Δ*cps* constructed as follows. A Δ*cps* PCR fragment, consisting of a kanamycin-resistance cassette flanked by *dexA* and *aliA* sequences, was amplified from *S. pneumoniae* strain D39(FP22) with primer pair FI4 and PE21 as described (41). This PCR product was introduced into *S. pneumoniae* by transformation, and kanamycin-resistant colonies, in which the entire capsule gene cluster was replaced with the kanamycin-resistance gene, were verified by PCR. For adherence assays, bacteria were grown to mid-exponential phase in Todd Hewitt broth supplemented with 5 g/L yeast extract (THY) and stored in 1-ml aliquots in THY containing 15% glycerol at -80°C. The number of colony forming units per milliliter (CFU/ml) was determined by plating serial 10-fold dilutions of test aliquots on Colombia blood agar plates. Before each assay, bacteria were thawed rapidly, washed once with RPMI 1640 medium without phenol red containing 1% FCS, and resuspended to the required CFU/ml in the same medium.

Purified pneumococcal polysaccharide type 2 powder was purchased from the American Type Culture Collection (ATCC, Manassas, VA), suspended in distilled water and used at a final concentration of 20 μg/ml.

Exposure of cells to *S. pneumoniae*. Adherence of pneumococci to epithelial cells was assayed essentially as described with a few modifications (25). Briefly, monolayers of Detroit 562 cells in tissue culture dishes (60 mm; samples for microarray analysis) or 6-well plates (35 mm; samples for time-curve) were washed twice with PBS, after which bacteria were added at a multiplicity of infection of 20:1 (bacteria:cells). Uninfected control cells were incubated with RPMI 1640 medium with 1% FCS only (mock-infected). After a predetermined incubation period (2h for microarray; 30 min, 2h, and 4 h for time-curve), nonadherent bacteria were removed by three washes with PBS. For quantification of adherence, epithelial cells were subsequently detached by treatment with 25% Trypsin, 1 mM EDTA in PBS and lysed by the addition of ice-cold 0.025% Triton X-100 in PBS. Serial 10-fold dilutions were plated on blood agar plates to count the number of adherent bacteria, and corrected mathematically to account for small differences in count in the initial inoculum.

RNA isolation. After washing, Detroit 562 cells were lysed directly in the tissue culture dish by addition of RLT buffer (Qiagen Benelux B.V.), collected with a rubber policeman, and transferred to a microcentrifuge tube. Subsequently, total RNA was isolated using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Total RNA was isolated from independent triplicate experiments. Quality and integrity of the purified RNA was verified by running all samples on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

Microarray hybridization. RNA was labeled using recommended protocols supplied by the manufacturer (Affymetrix**,** Santa Clara, CA). Briefly, double-stranded cDNA was synthesized from 6 µg of total RNA using a T7-oligo(dT) primer. The cDNA was purified

and converted to cRNA in an *in vitro* transcription reaction in the presence of biotinylated nucleotides. Subsequently, the biotinylated cRNA was purified and quantified by spectophotometric methods. cRNA yield was adjusted for carryover of unlabeled total RNA. Twenty micrograms of cRNA was fragmented for 35 min at 94°C, and hybridized to Affymetrix Human Genome U133 Plus 2.0 GeneChip arrays for 16 hours. After hybridization, the GeneChips were washed and stained using a GeneChip Fluidics Station 450 (Affymetrix**)**, and scanned with an Affymetrix GeneChip Scanner.

Microarray data analysis. The Affymetrix expression data were analyzed using the software package Gene Spring, version 7.3 (Agilent). All experiments (infected and mock-infected cells) were performed in triplicate to allow assessment of in-group variation. First, expression data obtained from infected cells was normalized to the median of the uninfected control samples. An initial selection of genes was made based on a 1.5-fold change in expression relative to that of uninfected control cells. Subsequently, an error model based on replicate measurements was defined in Gene Spring, and significance of differential expression was established using ANOVA and the false-discovery rate method (FDR) by Benjamini and Hochberg (5) to correct for multiple testing.

Transcriptional profiling of Detroit cells in response to adherence of *S. pneumoniae* strains was performed in three separate experiments. First, the response to adherent D39, D39Δ*cps*, and G54Δ*cps* was analyzed. Gene expression data of epithelial cells after adherence of wild-type *S. pneumoniae* strain G54 was collected second, and normalized to a separate set of uninfected control cells (obtained at the same time). The last experiment consisted of cells exposed to TIGR4, TIGR4Δ*cps*, low-dose D39Δ*cps*, purified type 2 capsular polysaccharides, and uninfected control cells used for normalization. In all cases, genes were

196 considered significantly up- or down regulated when $p<0.05$, and the average fold-change $\geq$
197 2.0 or ≤ 0.5, respectively.

198 Gene annotation and functional classification was based on information from the Gene
199 Ontology (4) or KEGG (20) databases. Significant overlap of specific sets of upregulated or
200 downregulated genes with KEGG pathways was assessed using GeneSpring.

201

202 **Real-time PCR.** Real-time quantitative PCR was used to validate selected data from
203 microarray experiments and to follow expression of a subset of genes over time. Total RNA
204 (2 μg) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit
205 according to the instructions provided by the manufacturer (Applied Biosystems, Foster City,
206 CA). Duplicate quantitative PCR assays were performed on 1 μl of 10x diluted cDNA using
207 TaqMan Gene Expression Assays (Applied Biosystems) of several target genes: ADRB2
208 (Hs00240532_s1), CXCL2 (Hs00601975_m1), DUSP5 (Hs00244839_m1), GAPDH
209 (Hs99999905_m1), IL6 (Hs00174131_m1), IL8 (Hs00174103_m1), MAP3K8
210 (Hs00178297_m1), MOAP1 (Hs00377893_g1), NFKBIA (Hs00153283_m1), TNFAIP3
211 (Hs00234712_m1), TRIB1 (Hs00179769_m1), and 18S (Hs99999901_s1). All assay were
212 done in 10-μl reactions containing TaqMan® Universal PCR Master Mix, 20X TaqMan®
213 Gene Expression Assay Mix, and cDNA on a Applied Biosystems 7500 FAST real-time PCR
214 system according to the manufacturer's instructions. The relative quantitation method ($\Delta\Delta C_T$)
215 (31) was used to evaluate quantitative variation in gene expression between a particular
216 experimental condition and control (uninfected) cells relative to each gene examined. The
217 GAPDH amplicon was used as the endogenous control for normalization of data.

218

219 **IL-6 ELISA.** Concentrations of IL-6 in Detroit 562 culture supernatants were measured
220 by a commercial ELISA kit (PeliKine Compact, Sanquin, Amsterdam, The Netherlands)

221 according to the instructions of the manufacturer. Levels of IL-6 were calculated based on
222 standards provided with the kit, and expressed as fold-increase relative to levels measured in
223 culture supernatants from uninfected control cells.
224
225 **Cytotoxicity assay.** As an indicator of cell viability during pneumococcal exposure,
226 released cytosolic lactate dehydrogenase (LDH) was quantified in cell culture supernatants
227 using the CytoTox96 Non-radioactive Cytotoxicity Assay kit (Promega, Madison, WI) as
228 recommended by the manufacturer.
229

RESULTS

Transcriptional response of epithelial cells exposed to *S. pneumoniae*. To be able to distinguish between a capsule-dependent and capsule-independent host response to pneumococcal adherence, we constructed an isogenic capsule-locus deletion mutant of wild-type, serotype 2-encapsulated *S. pneumoniae* strain D39. Consistent with earlier studies showing a negative influence of capsule expression on adherence and colonization ability of *S. pneumoniae* (17,24,53), the nonencapsulated derivative D39Δ*cps* displayed significantly higher *in vitro* adherence to epithelial cells compared to the D39 wild-type strain (Fig. 1). Further, pneumococcal adherence was found to increase in both a dose- and time-dependent manner (not shown).

Next, we used microarray analysis to characterize the transcriptional response of the human pharyngeal epithelial cells after 2 hours adherence of D39 and D39Δ*cps*. During this time frame, no adverse effect of pneumococcal exposure on cell viability was evident since no substantial LDH release was detected, while the level of adherence was at or near maximum (not shown). By selecting gene probes that showed at least a two-fold increase in expression compared to uninfected cells, a total of 136 probes representing 100 genes were found to be upregulated in Detroit cells in response to the two adherent *S. pneumoniae* strains (Fig. 2A, Supplementary Table S1). Of these, 19 were found to be induced in response both wild-type and Δ*cps* D39, whereas expression of the vast majority (72) appeared to be upregulated upon adherence of the wild-type strain only (Fig. 2A), indicative of a capsule-dependent set of host response genes. To determine whether this gene set is truly induced by the presence of encapsulated strains, or merely the result of suppression of expression of those particular genes by the higher number of adherent nonencapsulated bacteria, we used an additional, lower dose of D39Δ*cps* giving adherence levels equivalent to its encapsulated

wild-type (Fig. 1). Subsequent microarray analysis of the transcriptional response of the Detroit cells to low-dose D39Δ*cps* identified only four upregulated genes, none of which were identified with the previous two conditions (Supplementary Table S1). This clearly shows that the observed difference in response is not due to downregulation of gene expression by higher numbers of adherent nonencapsulated bacteria.

To examine if this particular expression profile was unique for the serotype 2 strain, or a more general capsule-dependent response, we included two additional, clinical isolates and their isogenic capsule mutants (Δ*cps*) in our analyses: serotype 19F-encapsulated G54, and TIGR4 of serotype 4. Again, the nonencapsulated derivatives showed greater levels of adherence compared to their wild-types in our *in vitro* assay (Fig. 1). Similar to our observations with D39, microarray analysis showed that the majority of the genes upregulated by adherent wild-type and Δ*cps* strains were induced only in response to the encapsulated strains (Figs. 2B and 2C, Supplementary Table S1). Strikingly, the presence of the serotype 4 strain triggered a much stronger response than the other two strains: almost three times as many genes were found to be upregulated. Even so, a considerable overlap was observed between the three sets of capsule-dependent response genes (Fig. 2D).

Evidently, the transcriptional response of pharyngeal epithelial cells to adherent pneumococci is greatly affected by capsule expression of the bacteria. To find out if this response requires the context of bacterial cells, we analyzed the expression pattern of Detroit cells after a 2-hour incubation with purified type 2 capsular polysaccharides. Only four genes showed an increase in expression of 2-fold or more: *IGFBP5*, encoding insulin-like growth factor binding protein 5; *CRISP3*, coding for cysteine-rich secretory protein 3; and two hypothetical ORFs (Supplementary Table S1). Although none of these loci were induced by the serotype 2-encapsulated D39, one of the hypotheticals was upregulated in response to

TIGR4 and TIGR4Δ*cps*, and the other three genes were also identified using the low-dose D39Δ*cps*.

Capsule-independent and capsule-dependent transcriptional responses. Twenty-two genes were identified that can be considered part of the common host response to pneumococcal infection, as they were induced by all genetic strain backgrounds, in most cases regardless of capsule (Table 1). The majority of these commonly induced genes encode proteins known to play a role in the immune response, such as those encoding several cytokines (IL-1β, IL-6), chemokines (IL-8, CCL20, CXCL1-3), and transcriptional regulators (NFκBIA, FOS). Other commonly induced genes included *EGR1*, involved in transcriptional regulation of cell growth and differentiation, and *BIRC3* and *IER3*, whose products are proposed to be involved in inhibition of apoptosis.

Over 300 genes were found to be induced specifically upon adherence of one or more of the wild-type strains D39, G54, and TIGR4. These genes can be considered part of either a capsule-dependent (i.e., induced by multiple encapsulated strains tested) or capsular serotype-dependent (i.e., upregulated specifically by exposure to one) host response. Genes upregulated in response to adherent encapsulated pneumococci were distributed among a variety of functional categories, but were clearly enriched for genes predicted to encode proteins involved in signal transduction and regulation of transcription (Table 2, Supplementary Table S1). No function could be assigned to approximately 25% of the transcripts (Table 2).

Notably, a significant number of genes were upregulated in response to all encapsulated strains that are part of the so-called mitogen-activated protein kinase (MAPK) signaling pathways (Table 3), which influence a variety of cellular processes such as cell growth, differentiation, and apoptosis in response to a wide range of stimuli (42). For example,

303 *ADRB2* and *EGFR* encode receptors that can trigger the MAPK cascade upon binding of
304 appropriate ligands, and several kinases and phosphates were identified that are involved in
305 subsequent transduction of signals from the cell membrane to the nucleus (Table 3). Several
306 of the other signal transduction molecules identified have been described to play a role in the
307 immune response. For example, the gene encoding the IL-1 receptor-associated kinase 2
308 (IRAK2) was induced specifically by D39; genes encoding two cytokines belonging to the
309 TNF ligand family (TNFSF18 and TNFSF9), and annexin 1 (ANXA1) by TIGR4; the
310 cytokine gene *LIF* and a suppressor of cytokine signaling (*SOCS1*) by both D39 and TIGR4;
311 and the gene coding for GTP-binding protein GEM was upregulated in response to all three
312 wild-type strains.

313 Close to 60 of the genes induced specifically in response to the encapsulated strains could
314 be classified as transcription factors. These are predicted to be involved in various biological
315 processes, such as immunoregulatory genes (*JUN*, Fos gene family members *FOSL1* and
316 *FOSB*, *PRDM1*, and *CEBPB*), and regulation of cell growth and differentiation (7 members
317 of the Krüppel-like factor (KLF) family). Several transcription factors were particularly
318 strongly induced by the presence of TIGR4 and/or D39: *ATF3*, identified as negative
319 regulator of TLR4 (14), and two other members of the early growth response family (*EGR2*
320 and *EGR3*). Other genes of interest included several apoptosis-related genes, and several
321 genes encoding extracellular matrix receptors (ITGAV, ITGB2, SDFR1) and a receptor-
322 ligand (PLAU, urokinase plasminogen activator), which was also found to be upregulated at
323 48 hr in a rat otitis media model (11).

324 Remarkably, more than half of the upregulated genes appeared to be capsular serotype-
325 specific, i.e., specifically induced by TIGR4 (Fig. 2D). In addition to the transcription factors
326 and signal transduction proteins mentioned above, predominant functional classes represented

in this gene set were transporters (e.g., 4 solute carrier family proteins), and cell proliferation (e.g., epithelial membrane protein EMP1) (Table 2).

To validate our microarray data, relative transcript levels were determined by quantitative real-time PCR on a selection of common and capsule-dependent host response genes, namely the innate immune genes *IL6*, *IL8*, *CXCL2*, *NFκBIA*, and *TNFαIP3*; the MAPK pathways genes *MAP3K8*, *DUSP5*, *ADRB2*, and *TRIB*; and apoptosis-related gene *MOAP1*. Overall, expression ratios obtained by microarray and real-time PCR analysis were concordant for all six conditions examined ($R^2 = 0.84$; Fig. 3).

To examine if changes observed in gene expression corresponded with changes in protein expression, we measured IL-6 in supernatants of Detroit cells after pneumococcal adherence. In correlation with the gene expression data, protein levels of IL-6 started to increase after 2 hr exposure of epithelial cells to all *S. pneumoniae* strains (not shown).

Expression of selected genes over time. The global transcriptional profiling described above focused on a two-hour time period of adherence. Based on the lists generated by these analyses, we selected a subset of five common innate immunity genes (see above) and followed their expression over time (30 min, 2h, and 4h) by real-time PCR upon adherence of the D39 and TIGR4 wild-type strains and their nonencapsulated derivatives (Fig. 4A). In addition, we examined expression of five capsule-dependent genes at the different time points (Fig. 4B). No difference in expression between uninfected control cells and infected cells was observed for the control 18S rRNA gene under any condition (data not shown). Expression of the common response genes was upregulated upon adherence in a time-dependent manner: no significant increase in expression compared to uninfected control cells at the earliest time point (30 min), while highest expression levels were achieved after 4h adherence (Fig. 4A). As expected, the response of these innate immunity genes to adherent encapsulated and

nonencapsulated strains was comparable. The same was true for one of the genes of the MAPK signaling pathway, *MAP3K8* (Fig. 4B), in agreement with the microarray results (Table 1). The other three MAPK genes showed a capsule-dependent expression pattern, particularly at the 2-hour time point and in response to the TIGR4 strain (Fig. 4B). However, both the difference in induction between encapsulated and nonencapsulated strains and the overall fold-change of genes was less pronounced than originally determined by the microarray analysis. Furthermore, no upregulation of the apoptosis modulating gene *MOAP1* could be demonstrated by real-time PCR under any condition (Fig. 4B), while it appeared to be induced 2-fold by TIGR4WT in our microarray analysis (Supplementary Table S1). For all genes examined, the presence of TIGR4 and TIGR4Δ*cps* resulted in the highest levels of expression changes, particularly at the latest time point. Interestingly, measurement of LDH release indicated that cell viability was also affected by the TIGR4 strains after 4h, while LDH release by cells infected with the D39 strains showed levels similar to the uninfected control cells (Fig. 5). Finally, we investigated the influence of low-dose D39Δ*cps* and purified type 2 capsular polysaccharides (20 and 50 μg/ml) on epithelial gene expression, but no upregulation was observed for any of the ten genes examined data not shown).

Genes downregulated in response to *S. pneumoniae*. Expression of 142 genes (represented by 150 gene probes) was found to be decreased at least two-fold upon adherence of wild-type and Δ*cps* D39 (Fig. 6, Supplementary Table S2). These repressed genes mainly belonged to metabolism, regulation of transcription, cell cycle, and, most predominantly, unknown functional categories (Table 4). Contrary to our observations made with upregulated genes, the vast majority (111) of repressed genes were downregulated only in response to the nonencapsulated D39Δ*cps*. In most cases, these genes showed a similar trend in response to strain D39WT, albeit to a lesser extent (e.g., 1.6-1.9 fold downregulation,

377 Supplementary Table S2). Interestingly, this response appeared to be specific for the D39
378 genetic background: only eight genes were found to be repressed by adherent G54 wild-type
379 or its nonencapsulated derivative G54Δ*cps*, no genes were downregulated in response to
380 TIGR4Δ*cps*, and twenty-one genes to TIGR4 wild-type (Fig. 4, Table 4). Eight genes were
381 downregulated in response to different strain backgrounds, among which *KRT4*, encoding
382 cytoskeletal protein keratin 4; *CLDN8*, encoding membrane protein claudin 8 involved in
383 cell-cell adhesion, and the anti-apoptotic gene *API5*.
384

DISCUSSION

The interplay between host and pathogen during the infectious process is complex and dynamic, requiring multiple factors from either side during different stages of infection. For the pneumococcus, colonization of the human nasopharynx is a prerequisite for infection (7). Colonization is initiated by adherence of *S. pneumoniae* to the host surface, influenced by several outer surface components, one of which is the pneumococcal capsule. For the host, the response tends to be focused on clearing the pathogen, mediated by activation of innate and adaptive defense mechanisms. A few studies have attempted to characterize the host response to pneumococci, both by using *in vitro* and *in vivo* models of infection (11,12,30,39,44). In this study, we examined the global transcriptional response of human epithelial cells to pneumococcal adherence *in vitro*, in particular in relation to the pneumococcal capsule.

A total of 322 genes were found to be upregulated in the human pharyngeal epithelial Detroit 562 cell line in response to adherent wild-type and nonencapsulated *S. pneumoniae* strains. In all cases, adherence of the encapsulated strains triggered a much greater transcriptional response than adherence of their isogenic nonencapsulated derivatives. This difference in gene induction does not appear to be associated with the number of adherent bacteria, as all wild-type strains adhered approximately one to two orders of magnitude less than their nonencapsulated mutants. Moreover, the results obtained with the lower dose D39Δ*cps* showed that this differential gene expression is indeed the consequence of upregulation of the genes in question in response to the encapsulated strain, not downregulation by the greater number of nonencapsulated bacteria attached to the cell surface. Detroit 562 genes induced by all pneumococcal strains tested included those encoding several cytokines, chemokines, and transcriptional regulators. Such transcriptional

409 profiles are consistent with a general innate immune response to bacteria mediated by Toll-
410 like receptor signaling, i.e., a stereotyped gene expression program (8). For *S. pneumoniae*,
411 activation of TLR2, TLR4, and TLR9 has been reported, by respectively pneumococcal cell
412 wall components, pneumolysin, and, most likely, CpG DNA (2,18,33,36,48,50). Since both
413 wild-type strains and their Δ*cps* derivatives contain these TLR ligands, stimulation of the
414 TLRs is likely to occur by all strains used in our experimental setup, resulting in the common
415 induction of, for instance, IL-6 gene expression and protein levels. Similar expression
416 profiles have been reported in other studies using various cell types and *in vivo* models,
417 underscoring the generality of this response. In respiratory epithelial cells, expression of IL-8
418 and CXCL2/3 (MIP-2ab) was shown to be increased in response to D39 (15,43). In a human
419 monocytic cell line, expression of IL-8, but not IL-1β, was found to be induced after 3 hours
420 exposure to pneumococci (44), while IL-1β and IL-6 production was observed in monocytes
421 after 24 hrs exposure (10). *In vivo*, levels of IL-1β and MIP-2ab were induced during early
422 pneumococcal otitis media in rats (11), while AP-1 family transcription factors were induced
423 both early and late during otitis media (11,30).

424 A large set of genes was found to be upregulated specifically upon adherence of the wild-
425 type strains, suggestive of a capsule-dependent host response. For the most part, distinct
426 genes sets were induced by serotype 2-encapsulated strain D39, serotype 19F-encapsulated
427 strain G54, and serotype 4-encapsulated strain TIGR4, indicating that the observed response
428 is largely capsular serotype-specific. The polysaccharide structures of capsular serotypes 2, 4
429 and 19F are very diverse (6), potentially explaining the limited overlap in the induced genes
430 sets. Incubation of Detroit cells with purified type 2 polysaccharides did not trigger the same
431 transcriptional response as seen with the encapsulated strains, indicating that the capsule is
432 best recognized in the context of (live) bacterial cells. Interestingly, adherence of the TIGR4
433 strain had the most pronounced effect on epithelial gene expression, suggesting that strain

genetic background is also of importance during this interaction. Recognition of pneumococcal capsule by the host is not mediated by TLRs. In mice, it has been described to depend on another PRR, namely the C-type lectin receptor (CLR) SIGN-R1 (homologue of DC-SIGN), expressed by mouse splenic and peritoneal macrophages (21). For immune activation to occur upon antigen binding by CLRs, simultaneous signalling through TLRs by appropriate ligands is believed to be required (27). SIGN-R1 has been shown to be essential for host resistance to both systemic and pulmonary infections with *S. pneumoniae* strains of different serotypes (21,28,29). However, expression of a homologue of this receptor on human epithelial cells has not been reported yet, and the exact role for this or other lectin-type receptors in mediating the capsule-dependent response observed in our experimental setup remains to be determined.

The encapsulated strains appeared to particularly induce several components of MAPK signaling pathways. The MAP kinases play pivotal roles in various cellular processes, including cell growth, inflammation, and apoptosis (42). Three major subfamilies of MAP kinases have been identified: extracellular signal-regulated kinases (ERK1/2), p38 MAP kinases, and c-Jun kinases (JNK). Several studies have demonstrated activation of both the JNK and p38 MAPK pathways in response to pneumococci, in bronchial and lung epithelial cells as well as endothelial cells (37,38,47). Our results are consistent with preferential activation of the JNK and p38 MAPK pathways. In human endothelial cells, activation of MAPK pathways by nonencapsulated pneumococci was shown to induce programmed cell death or apoptosis, executed by caspases 6 and 9 (38). Similarly, nonencapsulated *S. pneumoniae* induced caspase-6 dependent apoptosis of lung epithelial cells (46). In both cases, encapsulated strains mainly induced cell death in absence of DNA fragmentation, indicative of necrosis rather than apoptosis. When examining the transcriptional response of monocytes to pneumococci, Rogers *et al.* observed no significant effect on cell viability, but

459 did report upregulation of caspases 4 and 6 (44). Here, we found induction of expression of
460 several putative apoptosis-related genes, some of which have been described to induce
461 apoptosis in a caspase-dependent manner (e.g., *MOAP1*, *EGLN3*), caspases themselves were
462 not identified. Furthermore, no loss of cell viability was apparent during the 2-hour time
463 period of adherence used for microarray analysis, although extended (4h) adherence of the
464 TIGR4 strain did result in increased LDH release. For both endothelial and lung epithelial
465 cells, caspase-dependent apoptosis was only observed after 16 hours of pneumococcal
466 infection. It can, therefore, not be excluded that prolonged adherence will lead to apoptotic or
467 necrotic cell death of the pharyngeal epithelial cells in our setup as well. Apoptosis can
468 benefit both the pathogen, contributing to its virulence by inducing tissue injury, and the host,
469 enhancing bacterial killing and controlling the inflammatory response (as opposed to
470 necrosis). The induction of necrotic rather than apoptotic cell death by encapsulated strains
471 could potentially be mediated by expression of some of the genes identified as capsule- (or
472 serotype-) dependent in this study, but further experiments are needed to verify this
473 hypothesis.

474 A total of 161 genes were found to be downregulated in the epithelial cells. This response
475 appeared to be specific for the D39 genetic background, as very few genes were found to be
476 repressed by wild-type or nonencapsulated G54. Moreover, this did not appear to be mediated
477 by the capsule, as expression of the majority of genes was decreased upon adherence of the
478 nonencapsulated D39 derivative only. Possibly, the absence of capsule allows greater access
479 to other D39 surface components involved in eliciting this particular host response, an effect
480 likely to be enhanced by the greater number of adherent D39Δ*cps* compared to D39WT.

481 Adherence to and subsequent colonization of the nasopharyngeal epithelium is
482 indispensable for pneumococcal infection. By analyzing the *in vitro* transcriptional response
483 of human pharyngeal epithelial cells to pneumococcal adherence, we obtained evidence for a

484 subset of host genes specifically induced by encapsulated *S. pneumoniae* strains. *In vivo*, the
485 situation is likely to be complicated by the presence of multiple bacterial species at the
486 epithelial surface. For example, a synergistic increase in production of proinflammatory
487 cytokines was observed in response to costimulation with *S. pneumoniae* and of *Haemophilus*
488 *influenzae*, but *in vitro* by pharyngeal and lung epithelial cells (IL-8) and *in vivo* in the
489 murine nasopharynx (MIP-2) (43). Future studies, for instance using more extended periods
490 of adherence or exposure to mixed bacterial infections, will be required to exactly determine
491 the role of the pneumococcal capsule in triggering the host response. Furthermore, our data
492 suggest a central role for the TLR, lectin-type receptor, and MAP kinase pathways in defining
493 the host response to pneumococci, emphasizing the complexity of the host-pathogen
494 interaction, even at the earliest stages of the infectious cycle.
495

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REFERENCES

1. **Abeyta, M., G. G. Hardy, and J. Yother**. 2003. Genetic alteration of capsule type but not PspA type affects accessibility of surface-bound complement and surface antigens of *Streptococcus pneumoniae*. Infect. Immun. **71**:218-225.

2. **Albiger, B., S. Dahlberg, A. Sandgren, F. Wartha, K. Beiter, H. Katsuragi, S. Akira, S. Normark, and B. Henriques-Normark**. 2006. Toll-like receptor 9 acts at an early stage in host defence against pneumococcal infection. Cell Microbiol. **Epub ahead of print**.

3. **Albiger, B., A. Sandgren, H. Katsuragi, U. Meyer-Hoffert, K. Beiter, F. Wartha, M. Hornef, S. Normark, and B. H. Normark**. 2005. Myeloid differentiation factor 88-dependent signalling controls bacterial growth during colonization and systemic pneumococcal disease in mice. Cell Microbiol. **7**:1603-1615.

4. **Ashburner, M., C. A. Ball, J. A. Blake, D. Botstein, H. Butler, J. M. Cherry, A. P. Davis, K. Dolinski, S. S. Dwight, J. T. Eppig, M. A. Harris, D. P. Hill, L. Issel-Tarver, A. Kasarskis, S. Lewis, J. C. Matese, J. E. Richardson, M. Ringwald, G. M. Rubin, and G. Sherlock**. 2000. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat. Genet. **25**:25-29.

5. **Benjamini, Y. and Y. Hochberg**. 1995. Controlling the False Discovery Rate: a practical and powerful approach to multiple testing. J. Royal Statistical Society B **57**:289-300.

6. **Bentley, S. D., D. M. Aanensen, A. Mavroidi, D. Saunders, E. Rabbinowitsch, M. Collins, K. Donohoe, D. Harris, L. Murphy, M. A. Quail, G. Samuel, I. C. Skovsted,**

M. S. Kaltoft, B. Barrell, P. R. Reeves, J. Parkhill, and B. G. Spratt. 2006. Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. PLoS. Genet. **2**:e31.

7. **Bogaert, D., R. de Groot, and P. W. Hermans**. 2004. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. Lancet Infect. Dis. **4**:144-154.

8. **Boldrick, J. C., A. A. Alizadeh, M. Diehn, S. Dudoit, C. L. Liu, C. E. Belcher, D. Botstein, L. M. Staudt, P. O. Brown, and D. A. Relman**. 2002. Stereotyped and specific gene expression programs in human innate immune responses to bacteria. Proc. Natl. Acad. Sci. U. S. A **99**:972-977.

9. **Brueggemann, A. B., T. E. Peto, D. W. Crook, J. C. Butler, K. G. Kristinsson, and B. G. Spratt**. 2004. Temporal and geographic stability of the serogroup-specific invasive disease potential of *Streptococcus pneumoniae* in children. J. Infect. Dis. **190**:1203-1211.

10. **Cauwels, A., E. Wan, M. Leismann, and E. Tuomanen**. 1997. Coexistence of CD14-dependent and independent pathways for stimulation of human monocytes by gram-positive bacteria. Infect. Immun. **65**:3255-3260.

11. **Chen, A., H. S. Li, P. A. Hebda, A. Zeevi, and J. D. Swarts**. 2005. Gene expression profiles of early pneumococcal otitis media in the rat. Int. J. Pediatr. Otorhinolaryngol. **69**:1383-1393.

12. **Coimbra, R. S., V. Voisin, A. B. de Saizieu, R. L. Lindberg, D. Leppert, M. Wittwer, and S. L. Leib**. 2006. Gene expression in cortex and hippocampus during acute pneumococcal meningitis. BMC. Biol. **4**:15.

13. **Dopazo, J., A. Mendoza, J. Herrero, F. Caldara, Y. Humbert, L. Friedli, M. Guerrier, E. Grand-Schenk, C. Gandin, F. M. de, A. Polissi, G. Buell, G. Feger, E. Garcia, M. Peitsch, and J. F. Garcia-Bustos**. 2001. Annotated draft genomic sequence from a *Streptococcus pneumoniae* type 19F clinical isolate. Microb. Drug Resist. **7**:99-125.

14. **Gilchrist, M., V. Thorsson, B. Li, A. G. Rust, M. Korb, K. Kennedy, T. Hai, H. Bolouri, and A. Aderem**. 2006. Systems biology approaches identify ATF3 as a negative regulator of Toll-like receptor 4. Nature **441**:173-178.

15. **Graham, R. M. and J. C. Paton**. 2006. Differential role of CbpA and PspA in modulation of in vitro CXC chemokine responses of respiratory epithelial cells to infection with *Streptococcus pneumoniae*. Infect. Immun. **Epub ahead of print**.

16. **Gray, B. M. and H. C. Dillon, Jr.** 1988. Epidemiological studies of *Streptococcus pneumoniae* in infants: antibody to types 3, 6, 14, and 23 in the first two years of life. J. Infect. Dis. **158**:948-955.

17. **Hammerschmidt, S., S. Wolff, A. Hocke, S. Rosseau, E. Muller, and M. Rohde**. 2005. Illustration of pneumococcal polysaccharide capsule during adherence and invasion of epithelial cells. Infect. Immun. **73**:4653-4667.

18. **Han, S. H., J. H. Kim, M. Martin, S. M. Michalek, and M. H. Nahm**. 2003. Pneumococcal lipoteichoic acid (LTA) is not as potent as staphylococcal LTA in stimulating Toll-like receptor 2. Infect. Immun. **71**:5541-5548.

19. **Henrichsen, J.** 1995. Six newly recognized types of *Streptococcus pneumoniae*. J. Clin. Microbiol. **33**:2759-2762.

20. **Kanehisa, M., S. Goto, S. Kawashima, Y. Okuno, and M. Hattori**. 2004. The KEGG resource for deciphering the genome. Nucleic Acids Res. **32**:D277-D280.

21. **Kang, Y. S., J. Y. Kim, S. A. Bruening, M. Pack, A. Charalambous, A. Pritsker, T. M. Moran, J. M. Loeffler, R. M. Steinman, and C. G. Park**. 2004. The C-type lectin SIGN-R1 mediates uptake of the capsular polysaccharide of *Streptococcus pneumoniae* in the marginal zone of mouse spleen. Proc. Natl. Acad. Sci. U. S. A **101**:215-220.

22. **Kawai, T. and S. Akira**. 2006. TLR signaling. Cell Death. Differ. **13**:816-825.

23. **Khan, A. Q., Q. Chen, Z. Q. Wu, J. C. Paton, and C. M. Snapper**. 2005. Both innate immunity and type 1 humoral immunity to *Streptococcus pneumoniae* are mediated by MyD88 but differ in their relative levels of dependence on toll-like receptor 2. Infect. Immun. **73**:298-307.

24. **Kim, J. O. and J. N. Weiser**. 1998. Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of *Streptococcus pneumoniae*. J. Infect. Dis. **177**:368-377.

25. **Kloosterman, T. G., W. T. Hendriksen, J. J. Bijlsma, H. J. Bootsma, S. A. van Hijum, J. Kok, P. W. Hermans, and O. P. Kuipers**. 2006. Regulation of glutamine and glutamate metabolism by GlnR and GlnA in *Streptococcus pneumoniae*. J. Biol. Chem. **281**:25097-25109.

26. **Koedel, U., T. Rupprecht, B. Angele, J. Heesemann, H. Wagner, H. W. Pfister, and C. J. Kirschning**. 2004. MyD88 is required for mounting a robust host immune response to *Streptococcus pneumoniae* in the CNS. Brain **127**:1437-1445.

27. **Koppel, E. A., K. P. van Gisbergen, T. B. Geijtenbeek, and K. Y. van**. 2005. Distinct functions of DC-SIGN and its homologues L-SIGN (DC-SIGNR) and mSIGNR1 in pathogen recognition and immune regulation. Cell Microbiol. **7**:157-165.

28. **Koppel, E. A., C. W. Wieland, V. van den Berg, M. Litjens, S. Florquin, van Kooyk Y., van der Poll T., and T. B. Geijtenbeek**. 2005. Specific ICAM-3 grabbing nonintegrin-related 1 (SIGNR1) expressed by marginal zone macrophages is essential for defense against pulmonary *Streptococcus pneumoniae* infection. Eur. J. Immunol. **35**:2962-2969.

29. **Lanoue, A., M. R. Clatworthy, P. Smith, S. Green, M. J. Townsend, H. E. Jolin, K. G. Smith, P. G. Fallon, and A. N. McKenzie**. 2004. SIGN-R1 contributes to protection against lethal pneumococcal infection in mice. J. Exp. Med. **200**:1383-1393.

30. **Lin, J., Y. Tsuboi, W. Pan, G. S. Giebink, G. L. Adams, and Y. Kim**. 2002. Analysis by cDNA microarrays of altered gene expression in middle ears of rats following pneumococcal infection. Int. J. Pediatr. Otorhinolaryngol. **65**:203-211.

31. **Livak, K. J. and T. D. Schmittgen**. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25**:402-408.

32. **Magee, A. D. and J. Yother**. 2001. Requirement for capsule in colonization by *Streptococcus pneumoniae*. Infect. Immun. **69**:3755-3761.

33. **Malley, R., P. Henneke, S. C. Morse, M. J. Cieslewicz, M. Lipsitch, C. M. Thompson, E. Kurt-Jones, J. C. Paton, M. R. Wessels, and D. T. Golenbock**. 2003. Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. Proc. Natl. Acad. Sci. USA **100**:1966-1971.

611 34. **Mann, B., C. Orihuela, J. Antikainen, G. Gao, J. Sublett, T. K. Korhonen, and E.**
612 **Tuomanen**. 2006. Multifunctional role of choline binding protein G in pneumococcal
613 pathogenesis. Infect. Immun. **74**:821-829.

614 35. **Mitchell, T. J.** 2000. Virulence factors and the pathogenesis of disease caused by
615 Streptococcus pneumoniae. Res. Microbiol. **151**:413-419.

616 36. **Mogensen, T. H., S. R. Paludan, M. Kilian, and L. Ostergaard**. 2006. Live
617 *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* activate
618 the inflammatory response through Toll-like receptors 2, 4, and 9 in species-specific
619 patterns. J. Leukoc. Biol. **80**:267-277.

620 37. **N'Guessan, P. D., S. Hippenstiel, M. O. Etouem, J. Zahlten, W. Beermann, D.**
621 **Lindner, B. Opitz, M. Witzenrath, S. Rosseau, N. Suttorp, and B. Schmeck**. 2006.
622 *Streptococcus pneumoniae* induced p38 MAPK- and NF-kappaB-dependent COX-2
623 expression in human lung epithelium. Am. J. Physiol Lung Cell Mol. Physiol
624 **290**:L1131-L1138.

625 38. **N'Guessan, P. D., B. Schmeck, A. Ayim, A. C. Hocke, B. Brell, S. Hammerschmidt,**
626 **S. Rosseau, N. Suttorp, and S. Hippenstiel**. 2005. *Streptococcus pneumoniae* R6x
627 induced p38 MAPK and JNK-mediated caspase-dependent apoptosis in human
628 endothelial cells. Thromb. Haemost. **94**:295-303.

629 39. **Nelson, A. L., J. M. Barasch, R. M. Bunte, and J. N. Weiser**. 2005. Bacterial
630 colonization of nasal mucosa induces expression of siderocalin, an iron-sequestering
631 component of innate immunity. Cell Microbiol. **7**:1404-1417.

632 40. **Nelson, A. L., A. M. Roche, J. M. Gould, K. Chim, A. J. Ratner, and J. N. Weiser**.
633 2006. Capsule enhances pneumococcal colonization by limiting mucus-mediated
634 clearance. Infect. Immun. **Epub ahead of print**.

635 41. **Pearce, B. J., F. Iannelli, and G. Pozzi**. 2002. Construction of new unencapsulated
636 (rough) strains of *Streptococcus pneumoniae*. Res. Microbiol. **153**:243-247.

637 42. **Pearson, G., F. Robinson, G. T. Beers, B. E. Xu, M. Karandikar, K. Berman, and**
638 **M. H. Cobb**. 2001. Mitogen-activated protein (MAP) kinase pathways: regulation and
639 physiological functions. Endocr. Rev. **22**:153-183.

640 43. **Ratner, A. J., E. S. Lysenko, M. N. Paul, and J. N. Weiser**. 2005. Synergistic
641 proinflammatory responses induced by polymicrobial colonization of epithelial surfaces.
642 Proc. Natl. Acad. Sci. U. S. A **102**:3429-3434.

643 44. **Rogers, P. D., J. Thornton, K. S. Barker, D. O. McDaniel, G. S. Sacks, E. Swiatlo,**
644 **and L. S. McDaniel**. 2003. Pneumolysin-dependent and -independent gene expression
645 identified by cDNA microarray analysis of THP-1 human mononuclear cells stimulated
646 by *Streptococcus pneumoniae*. Infect. Immun. **71**:2087-2094.

647 45. **Rosenow, C., P. Ryan, J. N. Weiser, S. Johnson, P. Fontan, A. Ortqvist, and H. R.**
648 **Masure**. 1997. Contribution of novel choline-binding proteins to adherence, colonization
649 and immunogenicity of *Streptococcus pneumoniae*. Mol. Microbiol. **25**:819-829.

650 46. **Schmeck, B., R. Gross, P. D. N'Guessan, A. C. Hocke, S. Hammerschmidt, T. J.**
651 **Mitchell, S. Rosseau, N. Suttorp, and S. Hippenstiel**. 2004. *Streptococcus*
652 *pneumoniae*-induced caspase 6-dependent apoptosis in lung epithelium. Infect. Immun.
653 **72**:4940-4947.

654 47. **Schmeck, B., K. Moog, J. Zahlten, L. van, V, P. D. N'Guessan, B. Opitz, S. Rosseau,**
655 **N. Suttorp, and S. Hippenstiel**. 2006. *Streptococcus pneumoniae* induced c-Jun-N-
656 terminal kinase- and AP-1-dependent IL-8 release by lung epithelial BEAS-2B cells.
657 Respir. Res. **7**:98.

658 48. **Schroder, N. W., S. Morath, C. Alexander, L. Hamann, T. Hartung, U. Zahringer,**
659 **U. B. Gobel, J. R. Weber, and R. R. Schumann**. 2003. Lipoteichoic acid (LTA) of
660 *Streptococcus pneumoniae* and *Staphylococcus aureus* activates immune cells via Toll-
661 like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas
662 TLR-4 and MD-2 are not involved. J. Biol. Chem. **278**:15587-15594.

663 49. **Sjostrom, K., C. Spindler, A. Ortqvist, M. Kalin, A. Sandgren, S. Kuhlmann-**
664 **Berenzon, and B. Henriques-Normark**. 2006. Clonal and capsular types decide
665 whether pneumococci will act as a primary or opportunistic pathogen. Clin. Infect. Dis.
666 **42**:451-459.

667 50. **Srivastava, A., P. Henneke, A. Visintin, S. C. Morse, V. Martin, C. Watkins, J. C.**
668 **Paton, M. R. Wessels, D. T. Golenbock, and R. Malley**. 2005. The apoptotic response
669 to pneumolysin is Toll-like receptor 4 dependent and protects against pneumococcal
670 disease. Infect. Immun. **73**:6479-6487.

671 51. **Tettelin, H., K. E. Nelson, I. T. Paulsen, J. A. Eisen, T. D. Read, S. Peterson, J.**
672 **Heidelberg, R. T. DeBoy, D. H. Haft, R. J. Dodson, A. S. Durkin, M. Gwinn, J. F.**
673 **Kolonay, W. C. Nelson, J. D. Peterson, L. A. Umayam, O. White, S. L. Salzberg, M.**
674 **R. Lewis, D. Radune, E. Holtzapple, H. Khouri, A. M. Wolf, T. R. Utterback, C. L.**
675 **Hansen, L. A. McDonald, T. V. Feldblyum, S. Angiuoli, T. Dickinson, E. K. Hickey,**
676 **I. E. Holt, B. J. Loftus, F. Yang, H. O. Smith, J. C. Venter, B. A. Dougherty, D. A.**

677 **Morrison, S. K. Hollingshead, and C. M. Fraser**. 2001. Complete genome sequence of
678 a virulent isolate of *Streptococcus pneumoniae*. Science **293**:498-506.

679 52. **van Rossum, A. M., E. S. Lysenko, and J. N. Weiser**. 2005. Host and bacterial factors
680 contributing to the clearance of colonization by *Streptococcus pneumoniae* in a murine
681 model. Infect. Immun. **73**:7718-7726.

682 53. **Weiser, J. N., R. Austrian, P. K. Sreenivasan, and H. R. Masure**. 1994. Phase
683 variation in pneumococcal opacity: relationship between colonial morphology and
684 nasopharyngeal colonization. Infect. Immun. **62**:2582-2589.
685

685 **LEGENDS TO FIGURES**

686

687 **Figure 1. *In vitro* adherence of *S. pneumoniae*.** Adherence of wild-type *S. pneumoniae*
688 strains D39, G54, and TIGR4 and their isogenic nonencapsulated derivatives (Δ*cps*) to
689 Detroit 562 human pharyngeal epithelial cells. Data were obtained from three independent
690 experiments and presented as means ± standard errors of the mean.

691

692 **Figure 2. Distribution of genes upregulated in epithelial cells in response to adherent**
693 **pneumococci.** A. Distribution of epithelial genes induced by adherent serotype 2-
694 encapsulated D39WT and its nonencapsulated derivative D39Δ*cps*. B. Distribution of
695 epithelial genes induced by adherent serotype 19F-encapsulated G54WT and its
696 nonencapsulated derivative G54Δ*cps*. C. Distribution of epithelial genes induced by adherent
697 serotype 4-encapsulated TIGR4WT and its nonencapsulated derivative TIGR4Δ*cps*. D.
698 Distribution of epithelial genes induced by the three wild-type *S. pneumonia* strains. The
699 number of genes in each area within the Venn diagrams is indicated.

700

701 **Figure 3. Validation of microarray data by real-time PCR.** Ratios of transcript abundance
702 in infected versus uninfected cells obtained by microarray analysis (*x* axis) or real-time PCR
703 (*y* axis).

704

705 **Figure 4. Real-time PCR analysis of gene expression over time.**
706 Expression of specific genes (indicated at the top of each graph) was measured by real-time
707 PCR after predetermined time point of adherence (*x* axis). Log_2 ratios of infected cells/control
708 cells are the averages of two separate experiments. The dashed line indicates a two-fold
709 change in expression relative to uninfected control cells.

Figure 5. Cytotoxicity of epithelial cells upon adherence of pneumococci. Pneumococcal strains were allowed to adhere to Detroit 562 cells for time periods indicated on x axis after which cytotoxicity was assessed by LDH release.

Figure 6. Distribution of genes downregulated in pharyngeal epithelial Detroit 562 cells. Distribution of epithelial genes repressed by adherent wild-type strains D39WT, G54WT and TIGR4WT and the isogenic nonencapsulated derivatives D39Δ*cps* and G54Δ*cps*. The number of genes in each area within the Venn diagrams is indicated. The hatched ellipse represents genes repressed in response to G54Δ*cps*. No genes were found to be repressed in response to TIGR4Δ*cps*.

721 **Table 1. Common response genes in pharyngeal epithelial Detroit 562 cells during pneumococcal adherence[1].**

Locus	Description / Molecular Function	Fold-change[2] in response to:					
		D39WT	D39Δ*cps*	G54WT	G54Δ*cps*	TIGR4WT	TIGR4Δ*cps*
Immune Response							
IL-1β	Interleukin 1β	3.5	2.4	-[3]	2.2	2.3	1.5
IL-6	Interleukin 6	4.7	2.7	1.5	2.5	10.5	2.8
IL-8	Interleukin 8	9.6	5.8	3.1	5.8	8.4	3.8
CCL20	Chemokine (C-C motif) ligand 20	-	5.7	-	4.8	5.0	2.6
CXCL1	Chemokine (C-X-C motif) ligand 1	3.9	3.3	1.7	3.5	2.8	2.0
CXCL2	Chemokine (C-X-C motif) ligand 2	6.4	4.6	3.6	5.6	9.1	4.6
CXCL3	Chemokine (C-X-C motif) ligand 3	4.5	2.9	2.0	3.8	5.6	2.7
TNFαIP3	TNFα-induced protein 3; inhibits NF-κB activation	4.6	3.9	4.1	4.0	2.9	2.2
NFκBIA	Inhibits NF-κB by sequestering it in cytoplasm	2.1	2.1	2.5	2.2	2.6	2.3
NFκBIZ	Molecule possessing ankyrin repeats induced by LPS	2.7	2.6	2.6	2.5	2.5	2.1
FOS	Forms transcription factor AP-1 with JUN family members	13.0[4]	2.8	4.2	3.2	42.5	5.9
GEM	GTP-binding mitogen-induced protein	3.5	-	2.7	-	6.8	2.8
PTGS2	Prostaglandin-endoperoxide synthase	4.3	2.5	1.8	2.3	4.3	2.8
Signal Transduction							
HBEGF	Heparin-binding EGF-like growth factor	5.5	2.1	2.0	2.0	6.3	2.0
GDF15	Growth differentiation factor 15	4.2	2.3	-	2.0	6.0	2.0
MAP3K8	Mitogen-activated protein kinase kinase kinase 8	3.4	1.8	2.3	1.6	4.4	2.1
Regulation of Transcription							
EGR1	Early growth response 1	6.6	3.3	2.0	2.9	11.8	2.5
ZC3H12A	Zinc finger CCCH-type containing 12A	3.4	2.7	1.8	2.7	3.0	2.0
MYNN	Myoneurin	2.3	-	2.0	-	4.5	2.3
BHLHB2	Basic helix-loop-helix domain containing, class B, 2	2.3	-	2.3	-	5.6	2.7
Anti-apoptosis							
BIRC3	Baculoviral IAP repeat-containing 3	2.8	2.3	-	2.3	2.2	1.8
IER3	Immediate early response 3; induced by TNF	4.2	3.0	2.3	2.9	5.1	3.1

722 [1]Genes are classified as common if they show a fold-change ≥2 in response to all genetic strain backgrounds and at least 4 out of 6 conditions
723 [2]Average fold-change (FC) from three independent experiments; $p<0.05$
724 [3] -, not induced: FC<1.5
725 [4] $p=0.15$

Table 2. Functional categorization of pharyngeal epithelial Detroit 562 genes induced in response to adherent encapsulated pneumococci.

Global Classification	D39	G54	TIGR4	All 3	D39 and G54	D39 and TIGR4	G54 and TIGR4	Total
Apoptosis	0	0	5	1	0	4	2	12
Cell cycle, proliferation, and differentiation	0	3	19	1	0	3	1	27
Cytoskeleton organization and biogenesis	0	0	5	0	0	0	0	5
DNA metabolism	0	0	1	0	0	0	0	1
Extracellular matrix and adhesion	1	3	9	0	0	1	0	14
Immune response	1	0	4	7	0	5	1	18
Metabolism	1	3	6	0	0	1	2	14
Protein metabolism	0	1	1	0	0	1	0	5
Regulation of transcription	1	1	31	5	1	15	3	57
RNA metabolism	0	1	6	0	0	1	0	8
Signal transduction	2	5	18	5	0	20	5	55
Stress response	0	0	3	0	0	1	0	4
Transport and binding proteins	0	1	13	0	0	1	1	16
Unknown function	1	9	49	2	0	9	7	77
Total	7	27	170	21	1	62	22	311

Table 3. Capsule-dependent response genes in pharyngeal epithelial Detroit 562 cells during pneumococcal adherence:

MAPK Signaling Pathway.

Locus	Description / Molecular Function	Fold-change[1] in response to:					
		D39WT	D39Δ*cps*	G54WT	G54Δ*cps*	TIGR4WT	TIGR4Δ*cps*
ADRB2	β2-adrenergic receptor: Initiates activation of MAP kinases ERK1 and ERK2	2.3	-[2]	-	-	3.8	-
EGFR	Epidermal growth factor receptor	-	-	3.0	-	2.4	-
DUSP1	Dual specificity phosphatase 1: Inactivation of ERK2 by dephosphorylation	2.7	-	1.6	-	12.1	2.0
DUSP2	Inactivation of ERK1 and ERK2	2.3	-	-	-	4.9	-
DUSP4	Inactivation of ERK1, ERK2, and JNK	2.7	-	1.6	-	3.5	1.7
DUSP5	Inactivation of ERK1	3.8	-	2.2	-	7.0	1.7
DUSP6	Inactivation of ERK2	4.4	1.7	2.1	1.9	6.4	1.9
GADD45AB	Activation of MAPKKK activity (MEKK4 kinase)	2.4	-	-	-	2.7	-
MAPKAPK2	MAPK-activated protein kinase	-	-	2.2	-	-	-
MAP3K8	MAPKKK protein kinase: induces production of NF-κB	3.4	1.8	2.3	1.6	4.4	2.1
NR4A1	Nuclear receptor subfamily 4, group A, member 1	2.2	-	1.6	-	5.1	-
TRIB1	MAP2K1: activation of ERK1 and ERK2	2.2	-	1.6	-	6.7	1.8

[1]Average fold-change (FC) from three independent experiments; $p<0.05$

[2]-, not induced: FC<1.5

734 **Table 4. Functional categorization of pharyngeal epithelial Detroit 562 genes repressed in response to adherent pneumococci.**

Global Classification	D39	D39Δ*cps*	TIGR4	D39 and D39Δ*cps*	D39 and TIGR4	Total
Apoptosis	0	1	0	0	1	2
Cell cycle, proliferation, and differentiation	2	11	2	1	0	16
Cytoskeleton organization and biogenesis	0	4	0	2	0	6
DNA metabolism	0	4	1	0	0	5
Extracellular matrix and adhesion	0	5	0	3	0	8
Immune response	0	1	0	0	0	1
Metabolism	2	12	0	0	1	15
Nuclear export	0	1	0	0	1	2
Protein metabolism	0	4	2	1	0	7
Regulation of transcription	2	12	6	2	0	22
RNA metabolism	2	3	1	0	0	6
Signal transduction	1	8	0	0	0	9
Stress response	0	3	1	1	0	5
Transport and binding proteins	0	3	1	1	0	5
Unknown function	2	39	4	6	0	51
Total	11	111	18	17	3	160

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Figure 1.

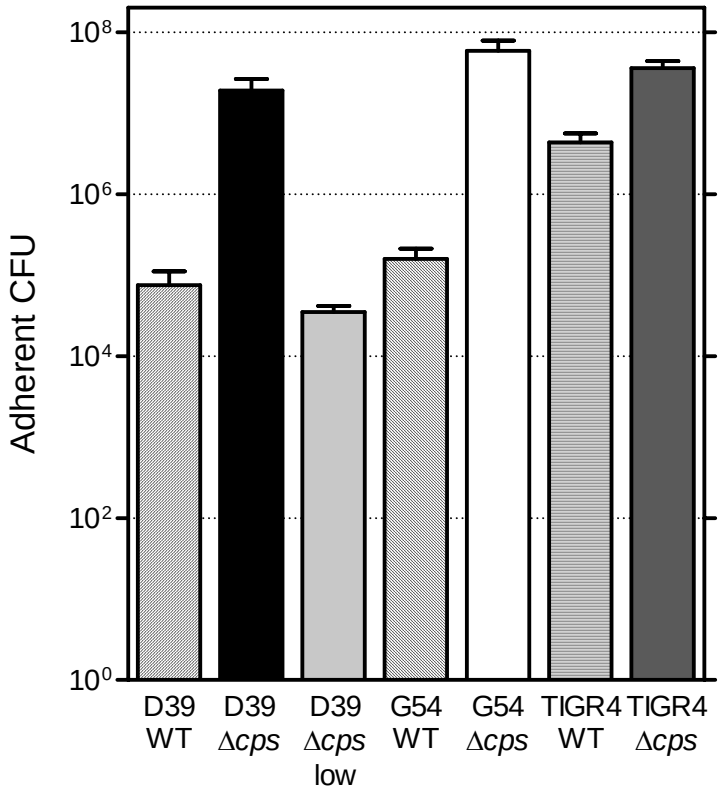

Adherent CFU
10^8
10^6
10^4
10^2
10^0
D39 WT
D39 Δcps
D39 Δcps low
G54 WT
G54 Δcps
TIGR4 WT
TIGR4 Δcps

Figure 2.

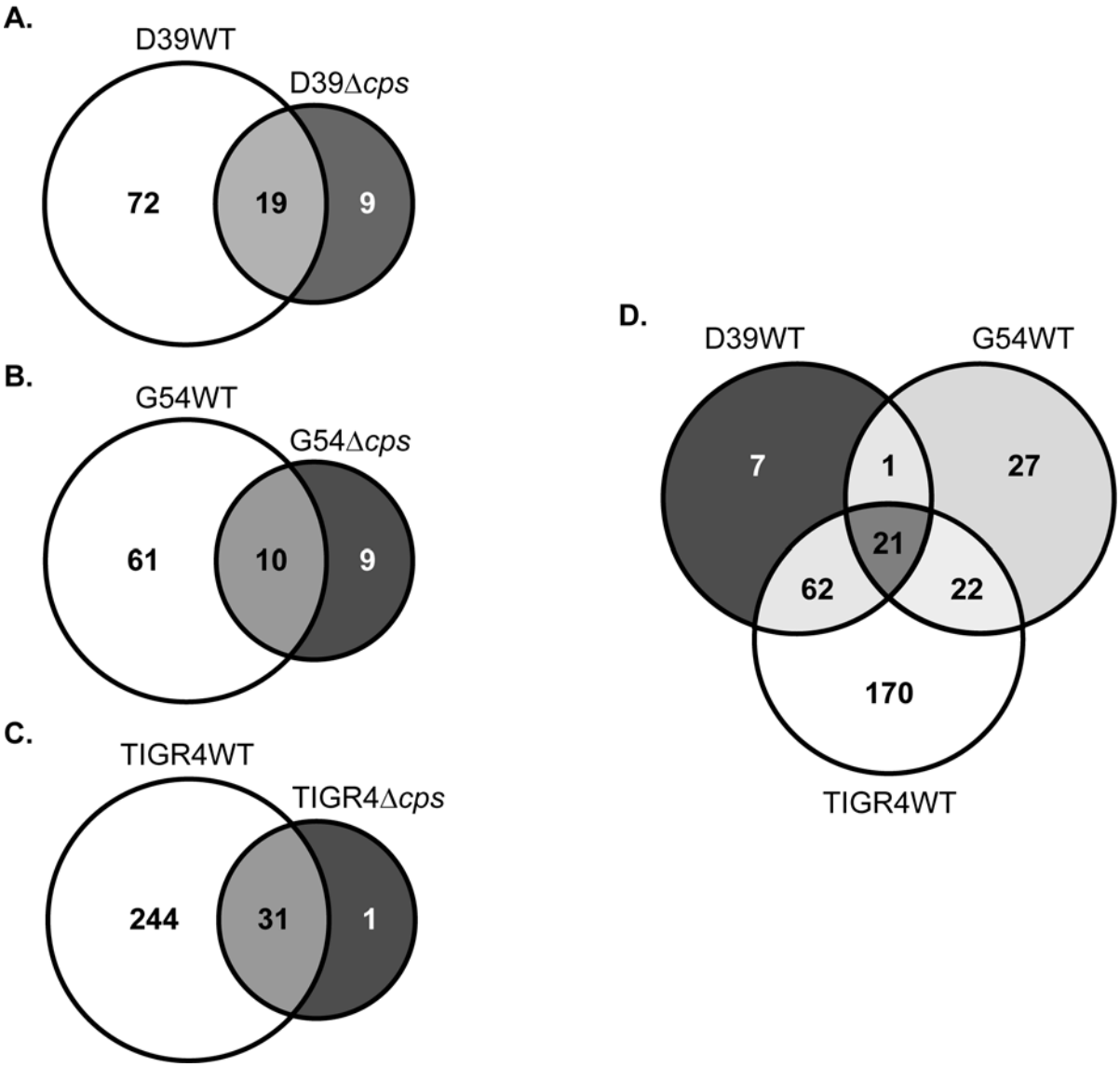

A.
D39WT
D39Δcps
72
19
9
B.
G54WT
G54Δcps
61
10
9
C.
TIGR4WT
TIGR4Δcps
244
31
1
D.
D39WT
G54WT
7
1
27
21
62
22
170
TIGR4WT

Figure 3.

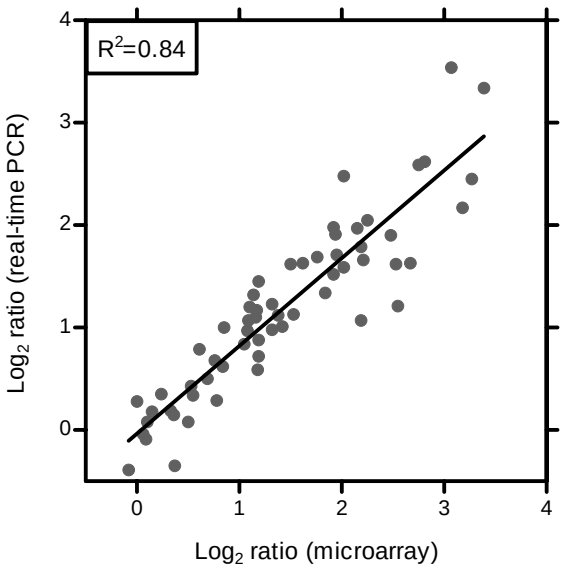
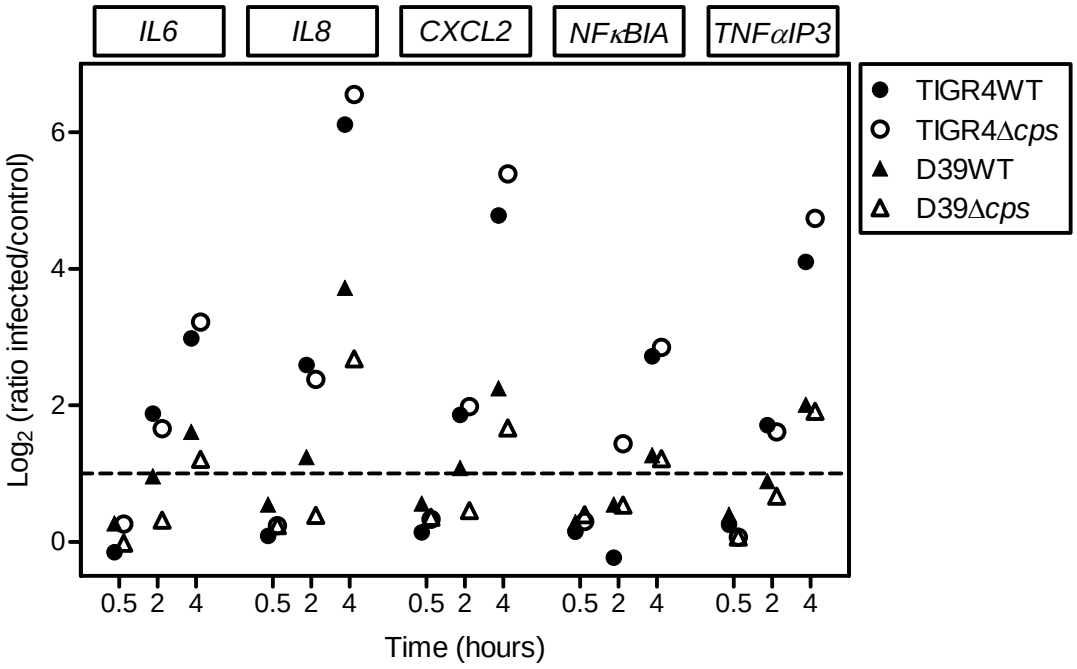


Figure 4.

A.


IL6
IL8
CXCL2
NFκBIA
TNFαIP3
TIGR4WT
TIGR4Δcps
D39WT
D39Δcps
Log2 (ratio infected/control)
0
2
4
6
0.5 2 4 0.5 2 4 0.5 2 4 0.5 2 4 0.5 2 4
Time (hours)

B.

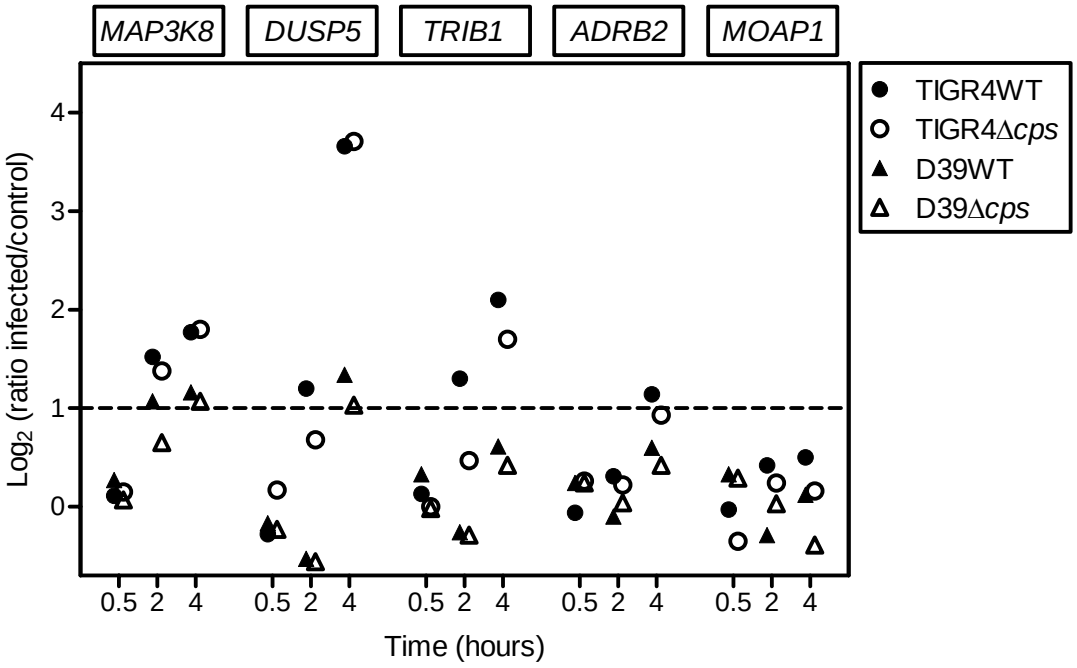

MAP3K8
DUSP5
TRIB1
ADRB2
MOAP1
TIGR4WT
TIGR4Δcps
D39WT
D39Δcps
Log2 (ratio infected/control)
0
1
2
3
4
0.5 2 4 0.5 2 4 0.5 2 4 0.5 2 4 0.5 2 4
Time (hours)

Figure 5.

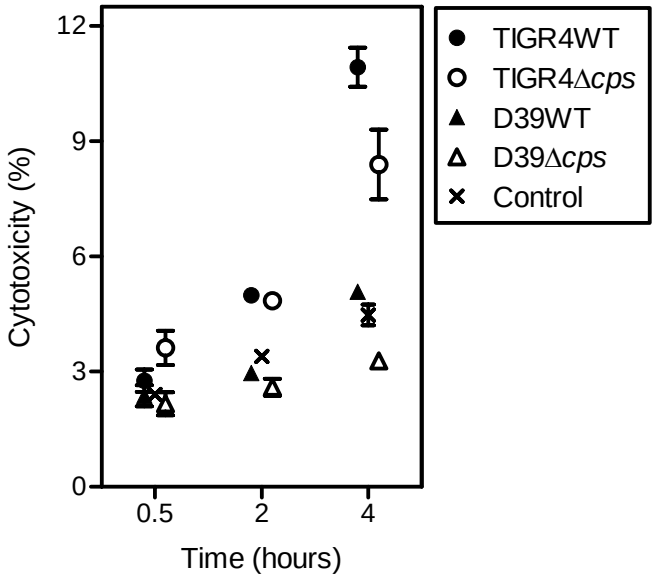

12
9
6
3
0
Cytotoxicity (%)
0.5
2
4
Time (hours)
TIGR4WT
TIGR4Δcps
D39WT
D39Δcps
Control

Figure 6.