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Transcriptome signature in young children with acute otitis media due to *Streptococcus pneumoniae*

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Abstract

Streptococcus pneumoniae (*Spn*) is the predominant causative organism of acute otitis media in children. To better understand the genes that are regulated at the onset of AOM caused by *Spn* infection in the middle ear, the transcriptome profile of peripheral blood mononuclear cells isolated from children prior to and during an AOM event was evaluated by microarray. We found that 1903 (6.2%) of 29,187 genes were differentially regulated greater than 2 fold at the onset of AOM compared to the pre-infection stage of the same children. The ontology of differentially regulated genes was dominated by those involved with the immune response. At onset of infection, genes associated with bacterial defenses were significantly up-regulated, including beta-defensin123, S100 protein A12, Toll-like receptor 5, IL-10, and those involved in the classical and alternative complement pathways. Genes associated with inhibition of bacterial entry through clathrin-dependent endocytosis were also up-regulated. In contrast, genes associated with cell-mediated immune responses were broadly down regulated. The results provide the first human transcriptome data identifying genes differentially regulated at the onset of AOM in children.

Keywords

STREPTOCOCCUS PNEUMONIAE; ACUTE OTITIS MEDIA; TRANSCRIPTOME PROFILE

1 Introduction

Acute otitis media (AOM) is the most frequent reason that children seek medical care for illness and receive antibiotics. AOM is associated with significant middle ear inflammation, mainly caused by bacterial infection. *Streptococcus pneumoniae* (*Spn*) is one of the most frequent pathogens isolated from middle ear fluid (MEF) of children with AOM [1]. The pathogenesis of AOM in humans involves nasopharyngeal (NP) colonization by the bacteria achieved by adherence and invasion of host NP epithelial cells. The bacterial inoculum in

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Authorship Contributions and Disclosure of Conflicts of Interest

The authors declare no competing commercial and financial interests. KL designed the study and performed all transcriptome experiments, analyzed the data and wrote the first draft of the paper. LC analyzed the data. RK performed the proteome experiments. MEP contributed to the design of the research, analyzed data and finalized writing of the paper.

the NP then refluxes up the eustachian tube near the adenoid tissues and gains entry to the middle ear. In *in vitro* experiments and an animal model, a robust inflammatory response with significant gene regulation has been described during *Spn* infection [2, 3] but data on human infection are needed.

Here we describe, for the first time in human children, an evaluation of the gene expression profile of peripheral blood mononuclear cells (PBMCs) associated with onset of AOM caused by *Spn*. This analysis was made possible during a prospective study on the immune responses of children to *Spn* during AOM with comparable PBMC samples taken within 4 weeks prior to onset of AOM. Double sampling from the same children reduced the complexities of analysis due to genetic heterogeneity among individuals. Closely timed sampling avoided the influence of changing age on responses in pediatric subjects. Our previous work identified significant changes in blood of a limited number of specific pro-inflammatory molecules during AOM caused by *Spn* [4,5,6]. However, with transcriptome analysis, we were able to identify a much broader range of significant changes in gene regulation.

Materials and methods

2.1 Subjects

The samples were collected from children (age 10-15 months) as approved by the IRB at Rochester General Hospital. The diagnosis of AOM was based on symptoms of fever, irritability or ear ache, and physical signs of inflammation of the tympanic membrane (bulging) with the presence of middle ear fluid (pus-laden fluid) documented by tympanocentesis. The inclusion criteria for children were: Caucasian children; pre-infection PBMC/serum sample within 4 weeks before the onset of AOM and the collection of an acute stage of illness PBMC/serum sample within 24 hours of onset of AOM; age 10-15 months at onset of AOM; AOM caused by *Spn* as proven by tympanocentesis; and moderate to severe AOM as judged by the physician. Exclusion criteria were: a history of immunodeficiency; chronic or recurrent AOM; any chronic disease; any other concurrent infectious disease; or receipt of steroids or other immunomodulatory agents within 1 month of any PBMC/serum sample.

2.2 Identification of pathogens

Identification of *Spn* in MEF was performed based on presence of α -hemolysis and inhibition of optochin and confirmed by a positive slide agglutination test according to established CLSI procedures (BBL™ Pneumoslide™ Kit, BD, Tetax, USA). *Nontypeable Haemophilus influenzae (NTHi)*, *Moraxella catarrhalis* and other otopathogens were identified as described previously [7]. The culture results were further verified by multiplex PCR as described previously [8].

2.3 RNA isolation

Four to ten milliliters of heparinized peripheral venous blood was collected for isolation of PBMCs and sera. PBMCs were isolated by Ficoll gradient and total RNA was extracted from PBMCs using a QIAamp RNA blood Mini Kit (Qiagen, Gaithersburg, MD) according to the manufacturer's instructions. Total RNA was treated with DNase I to remove contaminating DNA (Qiagen). RNA quality and integrity were determined utilizing an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Only high quality RNA, having a RIN of >7.0, and an A_{260}/A_{280} absorbance ratio of >1.8, was utilized for further experimentation.

2.4 Microarray analysis

DNA microarray analysis was performed in a blinded manner by Phalanx Biotech using the Human Whole Genome OneArray™ (Phalanx Biotech, Palo Alto, CA), which included 29,187 human genome probes and 1088 experimental control probes. RNA was converted to double-stranded cDNA and amplified using *in vitro* transcription that included amino-allyl UTP, and the aRNA product was subsequently conjugated with Cy5™ NHS ester (GEH Life sciences, Piscataway, NJ). Fragmented aRNA was hybridized at 42°C overnight using the HybBag mixing system with 1X OneArray Hybridization Buffer (Phalanx Biotech, Belmont, CA), 0.01 mg/ml sheared salmon sperm DNA (Promega, Madison, WI), at a concentration of 0.025 mg/ml labeled target. After hybridization, the arrays were washed according to the OneArray protocol. For the hybridization, the controls included dye intensity controls, positive controls containing a mixture of probes from 58 robustly expressed housekeeping genes and negative controls containing eight 60-mer oligonucleotide probes that do not cross-hybridize with human genomic probes on the Human OneArray DNA microarray. Gene expression was assessed in three replicates of each PBMC sample.

2.5 Quantitative real-time reverse transcriptase PCR analysis

Real time PCR was performed in our laboratory at the Rochester General Hospital Research Institute using a saved aliquot of the RNA preparation from the microarray experiments as templates. One hundred nanograms of total RNA was reverse transcribed to cDNA using an RT² First Strand Kit (QIAGEN). Detection and quantification of gene expression in PBMCs was performed using an RT² Profiler Human Custom Kit according to the manufacturer's instructions (QIAGEN). Quantitative real-time reverse transcriptase PCR was performed using a CFX 96 Thermocycler (Bio-Rad, Fairfield, CA). The threshold and baseline were set automatically using the pcr/array analysis method according to the manufacturer's instructions (SABiosciences). C_T data were uploaded into the data analysis template on the manufacturer's website (<http://www.sabiosciences.com/pcr/arrayanalysis.php>). The relative expression of genes compared with the expression in control samples was calculated on the website using the $\Delta \Delta C_T$ method with five housekeeping genes as controls.

2.6 Proteome assays

The protein levels of S100A12 in the serum and MEF samples were determined from the same children where microarray was performed using the CircuLex S100A12 ELISA Kit according to standard instructions in the protocol. IL-10 levels were determined in the same samples using a Bio-Plex Pro Assay (Bio-Rad Laboratories, Hercules, California, USA) according to manufacturer's instructions and read with a Luminex instrument.

2.7 Data Analysis

Primary data analysis was performed by Phalanx Biotech. Raw intensity signals for each microarray were captured using a Molecular Dynamics™ Axon 4100A scanner, measured using GenePixPro™ Software, and stored in GPR format. The data from all microarrays in each experimental set was then passed to Rosetta Resolver (Rosetta Biosoftware, Cambridge, MA) for analysis. Testing was performed by combining technical replicates (<http://www.phalanxbiotech.com>) and performing statistical analyses using Rosetta Resolver's proprietary modeling techniques (<http://www.rosettabio.com/products/resolver>). The gene intensities were adjusted by subtracting the array background, and then normalized to housekeeping genes (four probes per gene were selected from seven robustly expressed housekeeping genes). The data with a consistent and high quality readout verified by quality control at Phalanx Biotech of microarray were analyzed at the Rochester Institute of Technology (L.C.) for biological function groupings. The data from 12 hybridizations of 4

children were pooled and the fold change was calculated by comparing the readout at onset of AOM compared to the pre-infection healthy state. (4 children at 2 stages = 8 data sets and for each stage and triple hybridizations were performed; therefore, each stage -AOM or healthy- had 4 samples \times 3 hybridizations yields 12 hybridizations for each stage.) Genes with greater than a two-fold increase or decrease were considered significant and included for further analysis. The biological themes associated with these genes were analyzed using DAVID software [<http://david.abcc.ncifcrf.gov>]. To identify lexical enrichments, the terminology defined by the Gene Ontology (GO) Consortium [<http://www.geneontology.org>] was used.

3 Results

3.1 Transcriptome responses in children with AOM due to *Spn*

1903 (6.2%) of 29,187 genes assessed were differentially expressed 2-fold up or down in all 4 of the studied children at the onset of AOM compared to their pre-infection stage. Of the 1903 genes differentially regulated, 970 transcripts (51% of the total) were significantly up-regulated during AOM compared to the pre-infection state and 933 transcripts (49 % of the total) were significantly down-regulated (Table 1). The complete list of differentially expressed genes is provided in supplementary Table 1 and accession number GSE23140.

3.2 Gene functional clustering is dominated by the immune response

To understand the biological functions of the genes differentially expressed at the onset of AOM, the 1903 transcripts identified above were analyzed with DAVID v6.7 software. It was found that the top-ranked gene ontology was typical of an immune response to bacterial infection, followed by the clusters of regulation of transcription, cell morphogenesis and cellular homeostasis (Table 2). Further analysis found that there were 127 genes that could be grouped in the host immune defense category which could be further classified into the subgroups of innate immune response, leukocyte activation and differentiation, response to bacteria infection, regulation of apoptosis, kinase activity and biosynthesis.

3.3 Differentially expressed genes related to innate immunity

Since the understanding of the immune response mechanism in children is still incomplete, our analysis of the transcriptome profile was focused on the 127 transcripts of host immune defense responses. We found that 19 genes were classified to innate immune responses which included genes for the classical and alternative complement pathway, toll-like receptors and interleukins.

Of the genes involved in the classical complement pathway, the highest up-regulated gene was C1QA and C1QB. In addition, C1S was down regulated significantly and CFD, a component of the alternative complement pathway with function similar to C1S in the classical pathway, was up regulated (Table 3).

Surprisingly, there were not many pathogen-specific-receptors differentially expressed. TLR2, a common receptor up-regulated in *Spn* infection in rodent models, did not show differential expression at onset of *Spn* infection in the middle ear in the children. However, TLR5 was significantly up-regulated and TLR10 showed significant down-regulation (Table 3).

3.4 Differentially expressed genes related to anti-bacterial response

Among the 127 genes related to host immune defense responses, 8 genes associated with anti-bacterial activity were differentially expressed at onset of AOM. The highest up-regulated genes were beta-defensin 123 (DEFB123), S100 proteins S100A12 and S100A9,

and HIST1H2BJ (a broad antibacterial molecule) (Table 3). Other genes related to bactericidal activity towards Gram-positive bacteria were down-regulated such as DEFB132 and lactotransferrin (LTF).

3.5 Differentially expressed cytokine genes

Genes associated with 16 cytokines were differentially expressed at onset of AOM, including 5 interleukins and 8 chemokines (Table 3). Among the 8 up-regulated genes, the highest expression was IL-10, which plays an important role in inhibition of CD4 T-cell activation (except its antibacterial activity). CCL5, associated with chemotaxis of T cells was up-regulated 3.9 fold. CCL1, with chemotactic activity for monocytes but not for neutrophils, was up-regulated 4.8 fold. CCL28, chemotactic for resting CD4 or CD8 T cells and eosinophils, was up-regulated 4.6 fold. IL1F8, involved in the inflammatory response, was up-regulated 2.9 fold. Down-regulated genes included CCL20, a chemotactic factor for lymphocytes, neutrophils and dendritic cells towards an infection site, IL-2, required for T-cell proliferation and other activities crucial to regulation of the immune response, and IL-1 β , involved in inflammatory reactions. In short, the regulated genes associated with cytokines were consistent with enhancement of antibacterial activity and inhibition of cell mediated immune responses.

3.6 Differentially expressed genes related to cellular immunity

22 differentially expressed genes were grouped to lymphocyte activation and differentiation. Fifteen of them showed down regulation at the onset of *Spn* AOM. The most predominant down regulated genes were CD80, which is involved in antigen presentation and T-lymphocyte activation, and CD74, which plays a critical role in MHC class II antigen processing. Among 7 up regulated genes there were 3, including IL10, ADORA2B and VSIG4 that play an important role in inhibition of lymphocyte activation and proliferation (Table 3). In addition, among the genes associated with B-cells activation and proliferation, CD19, SWAP70, CD79A, BLNK, and FOXP1 were all down-regulated.

3.7 Differentially expressed genes indicative of endocytosis

We found many genes involved in the clathrin-mediated endocytosis cascade were differentially regulated, including down regulation of CD2 associated protein (CD2AP) and Cb1-interacting protein of 85 kDa (CIN85), consistent with an effect whereby the entrance of *Spn* into host cells was inhibited. Other differentially expressed genes in this cascade included PLD, LDLR, ARH, FIP, RAB11, RAB4, RAB5, AMSH, EEA1 and MVB12. In contrast, no genes involved in the clathrin-independent endocytic machinery were differentially regulated (Fig. 1).

3.8 Data verification

To support the microarray data, we performed qRT-PCR analysis for a subset of genes with different biofunctional categories, including IL-1 receptors (IL-1R1), cytokines (IL-10), chemokines (CCL5), antibacterial molecules (S100A9, PGLYRP1), innate immune response (TLR10), B-cell activation (BLNK) and T-cell activation (CD3g). Our qRT-PCR data were consistent with the data generated with microarray (Fig. 2).

3.9 Individual sample analysis

Although the timing of PBMC samples and the study children were carefully selected to allow pooling of data, sources of variability not strictly genetically controlled may have confounded the results. To further strengthen our array data, the transcriptional changes in 17 genes for each child were analyzed (Fig. 3). Similar levels of gene up-regulation and down-regulation were seen with no differences in the functional clusters. These data are in

agreement with our array data and further support the transcriptome changes we found associated with the onset of AOM compared to their healthy pre-infection stages.

3.10 Proteome analysis

To confirm that the transcriptome results were reflected in the proteome, we evaluated concentrations of S100A12 and IL-10 proteins in the sera and MEF of the study children. Similar to the transcriptome results, the levels of S100A12 in the serum samples of the 4 children at onset of AOM (32.5 ± 4.5 ng/ml) were significantly higher ($p=0.04$) compared to when the children were healthy (11.3 ± 2.5 ng/ml). Interestingly, the levels of S100A12 in the MEF, at the site of infection, were about 450-fold higher (14677 ± 1435 ng/ml) than serum in the AOM stage. Similarly, the levels of IL-10 in serum samples of children at onset of AOM (4.8 ± 0.9 pg/ml) were significantly higher ($p=0.04$) compared to when the children were healthy (2.9 ± 0.3 pg/ml). IL-10 in the MEF was higher (314 ± 194 pg/ml) at the site of infection in the AOM children over serum during infection.

4. Discussion

To our knowledge, this is the first report on transcriptome changes associated with *Spn* infection of the middle ear in children. The genes associated with the immune response ontology were dominant and could be further classified into the subgroups of innate immune response, leukocyte activation and differentiation, and response to bacteria infection. To our surprise, genes associated with cell mediated immune response were significantly down-regulated in the studied children. In addition, genes associated with the inhibition of bacterial entry to host cells through clathrin-dependent endocytosis were down-regulated.

4.1. Number of genes mobilized during AOM due to *Spn* in young children

Overall, we found that 6.2% of 29,187 genes evaluated were differentially expressed greater than 2 fold in all four children with AOM caused by *Spn* compared to their healthy stage. The percentage of genes regulated in AOM children upon *Spn* infection is consistent with results from children with other infections and models of infection. For example in a study of children infected with *influenza virus*, 1.3% of the studied genes were differentially expressed in the acute phase of infection [9]. In another study by our group involving other children with AOM caused by *NTHi*, 4.8% of the studied genes showed a > 2 fold change compared to the pre-infection healthy stage [10]. Using an *in vitro* model, the transcriptional response of RAW 264.7 cell's to *Salmonella typhimurium* infection involved 5.8% (34/588) of genes studied using a 4-fold or greater change as a cut off [11]. Using a microarray of the human genome, 1.5% (22/1506) of host genes were identified as induced by *Pseudomonas aeruginosa* infection of A549 cells [12].

4.2. Up-regulation of antibacterial response genes predominate in children with AOM due to *Spn* infection

We found the genes related to antibacterial activity, such as S100 proteins, defensins, cytokines/chemokines, complement and Toll like receptors were particularly up-regulated at onset of AOM due to *Spn*. We confirmed that the transcriptome signature was reflected in the proteome for S100A12 in serum and at the site of infection in the middle ear. In agreement, we had previously observed an increase in S100A12 in serum of children at onset of AOM and a return to pre-infection levels with recovery from AOM [5]. However the up-regulation of DEFB123 and BPI at onset of the AOM stage in young children is a new finding and indicates that these additional specific antibacterial responses are mobilized as part of the innate defense at onset of the infection of *Spn* in the middle ear. It is known that defensins are a family of secreted antimicrobial peptides directly interfering with bacterial membranes in the innate immune response. DEFB123 exerts antimicrobial activity

against Gram-positive bacteria [13]. Administration of a derivative of bactericidal permeability-increasing protein (BPI) provided protection against *Spn* infection in a mouse model [14], and S100A12 had a direct antimicrobial effect on bacteria [15].

The role of complement in eradication of *Spn* is a well-known immune defense mechanism. Therefore, finding that genes for the classical complement pathway were up-regulated in young children at onset of AOM due to *Spn* was expected. Using strains of mice with genetic deficiencies of complement components, Brown et al found that the classical pathway is the dominant pathway for activation of the complement system during innate immunity to *Spn* [16]. Our study is consistent with the mouse model observation. However, surprisingly, we also found that complement factor D gene (CFD), encoding a protein in the alternative complement pathway, was also up-regulated at the onset of AOM, suggesting activation of the alternative pathway as well. The result suggests the need for further study of the role of the alternative pathway in the innate response to *Spn*.

Toll-like receptors are transmembrane proteins expressed by cells of the innate immune system that recognize invading microbes and activate signaling pathways that launch immune and inflammatory responses. The TLR2 receptor has been classically described as the main receptor activated by *Spn*. However, recently, Muñoz et al [17] reported that flagellin of *Spn* could locally activate innate immunity against *Spn* as flagellin signals through TLR5. In the current study, we found that TLR5 was up-regulated significantly in children with AOM caused by *Spn*. Unexpectedly, we found that TLR10 was down regulated. Human TLR10 is an orphan member of the TLR family and its exact function is not known. However, TLR signaling negatively regulated by various proteins has been reported previously [18]. Further study of the role of TLR5 and TLR10 during *Spn* infection may be warranted.

4.3. Down regulation of cell-mediated immune response in young children at onset of AOM caused by *Spn*

The current study indicated that the genes associated with the cellular immune response to infection were down regulated at onset of AOM as demonstrated by the indirect cytokine gene regulation and direct cell proliferation and activation regulation. Specifically, cytokine IL-2 and IL-1 β were down-regulated. It is known that IL-2 is required for T-cell proliferation as well as other activities crucial to the regulation of the immune response. IL-1 β is an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation and differentiation. The observed down-regulation of IL-1 β at onset of AOM is consistent with the weak cellular immune response in young children that was observed [19]. We confirmed that the transcriptome signature was reflected in the proteome for IL-10 in serum and at the site of infection in the middle ear.

4.4. Endocytosis of bacteria by host cells in young children

Endocytosis is a process used by cells to engulf bacteria [20]. We found that many genes associated with endocytosis were differentially regulated in children with AOM due to *Spn*. The genes all belonged to the clathrin-mediated endocytosis group. The platelet-activating factor receptor (PAFR) gene was up-regulated and PAFR has been shown to be a receptor mediating entry of *Spn* into cells [21]. Data from studies using drugs that inhibit endocytosis support a role for clathrin-dependent endocytosis in the entry of *Spn* into cells [21,22]. In one of those studies, uptake of the *Spn* vacuole involved clathrin, and half the bacteria proceeded into vacuoles marked by Rab5 and Rab7 [21]. Our study showed significant regulation of genes encoding Rab5 and Rab7 at onset of AOM due to *Spn* infection.

We also found that genes for both CD2 associated protein (CD2AP) and Cb1-interacting protein of 85 kDa (CIN85) were significantly down-regulated. Those genes encode proteins necessary for ligand-induced endocytosis of receptor tyrosine kinases during bacterial entry. A previous study found that decreased expression of these proteins strongly inhibited *Listeria monocytogenes* entry into host cells [23]. Therefore, the down-regulation of these genes in the current study indicates that host cells in children with AOM may have defects in antigen presentation by antigen presenting cells.

4.5. General conclusions, limitations of study results and future directions

The remarkable power of microarray analysis is demonstrated in this study of gene regulation associated with a common pediatric infection caused by respiratory bacteria. With a focus on the immune response genes, we found genes related to antibacterial activity, such as S100 proteins, defensins, cytokines/chemokines, complement and Toll like receptors were particularly up-regulated as expected. The regulation of genes related to DEFB123 and BPI, the alternative complement pathway, TLR5 and TLR10, 5 interleukins and 8 chemokines with functions of enhanced antibacterial activity and inhibition of lymphocyte activation and differentiation and the clathrin-mediated endocytosis cascade are new findings.

Limitations of our study include those inherent in clinical studies in humans of gene regulation where genetic heterogeneity, timing of exposure to pathogens, and dose and strain of the pathogen. To reduce the heterogeneity of the samples, we selected samples from children of very similar age, of the same race, medical history record, pathogen in the middle ear and severity of disease. In addition, to avoid the technical error and subjectivity in microarray assay interpretation, the data was blinded and compiled by an outside laboratory (Phalanx Biotech). We used PBMCs as the source of clinical material because blood is the reservoir for immune cells that migrate to the middle ear during AOM. Leukocytes isolated from the peripheral blood constitute an accessible source of clinically relevant information from which a comprehensive molecular phenotype can be obtained by microarray analysis [24-29].

Future directions of our work include analysis of the gene regulation and protein products involved in the innate and adaptive immune response to *Spn* infection and the paradoxical results regarding the cell mediated immune response. The regulation of genes involved in the clathrin-dependent endocytosis cascade also should be further understood. Thus, the current results provide valuable information for further identifying new targets for study to understand the AOM disease process and direct us to development of intervention and therapeutic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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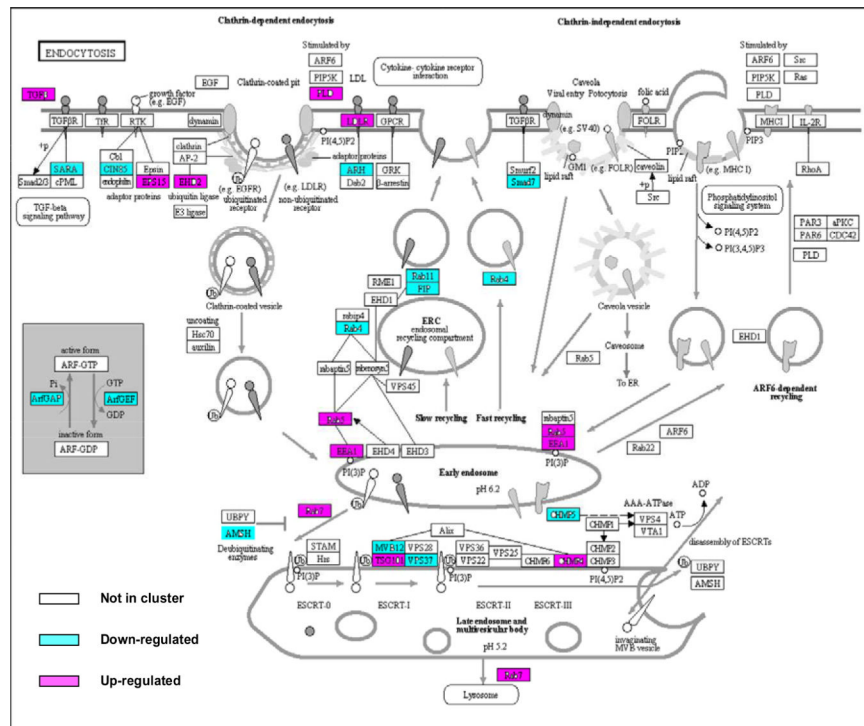


Fig. 1. Gene expression in 4 children with AOM due to *Spn* is enriched in genes involved in clathrin-dependent endocytosis
 Fold change in gene expression from children with AOM due to *Spn* versus their pre-infection healthy stage is shown on the KEGG pathway map (modified from map04144, at the Kyoto Encyclopedia of Genes and Genomes [KEGG] website <http://www.genome.jp/kegg/kegg1.html>). The genes are colored according to the transcript with the fold change in expression. Red color indicates up-regulation at onset of *Spn* infection, green down-regulation.

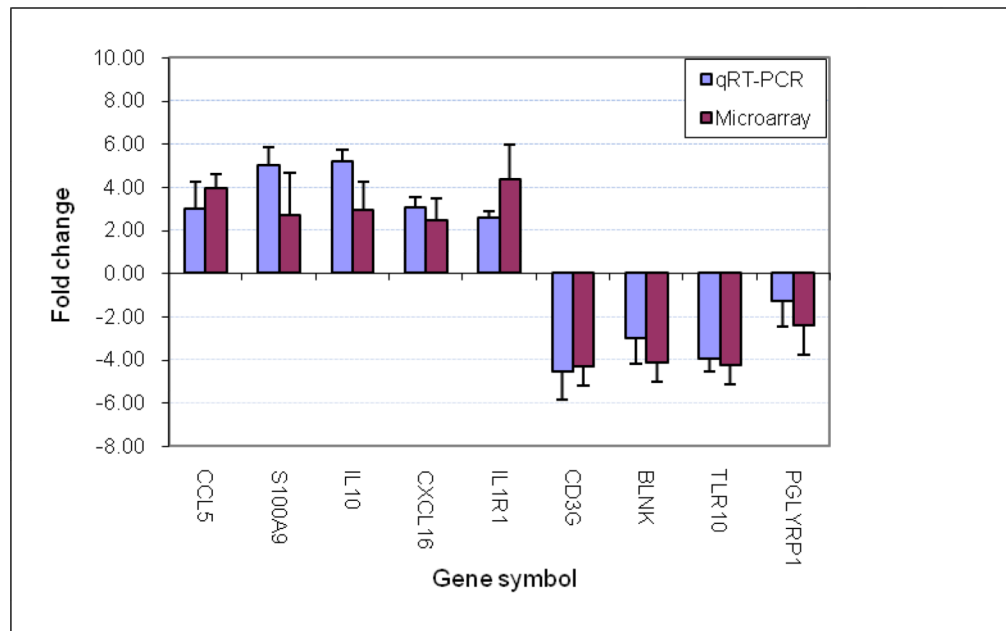


Fig. 2. Comparison of genes regulated as measured by microarray and qRT-PCR

The fold change of gene expression was derived by comparison of the gene expression at the onset of AOM due to *Spn* (AOM) with the pre-infection healthy stage (Preinf) in the same children with the same samples. The data from the 4 children are presented as a mean \pm SD. In all the genes studied there was no difference for the fold change between qRT-PCR and microarray ($p>0.05$).

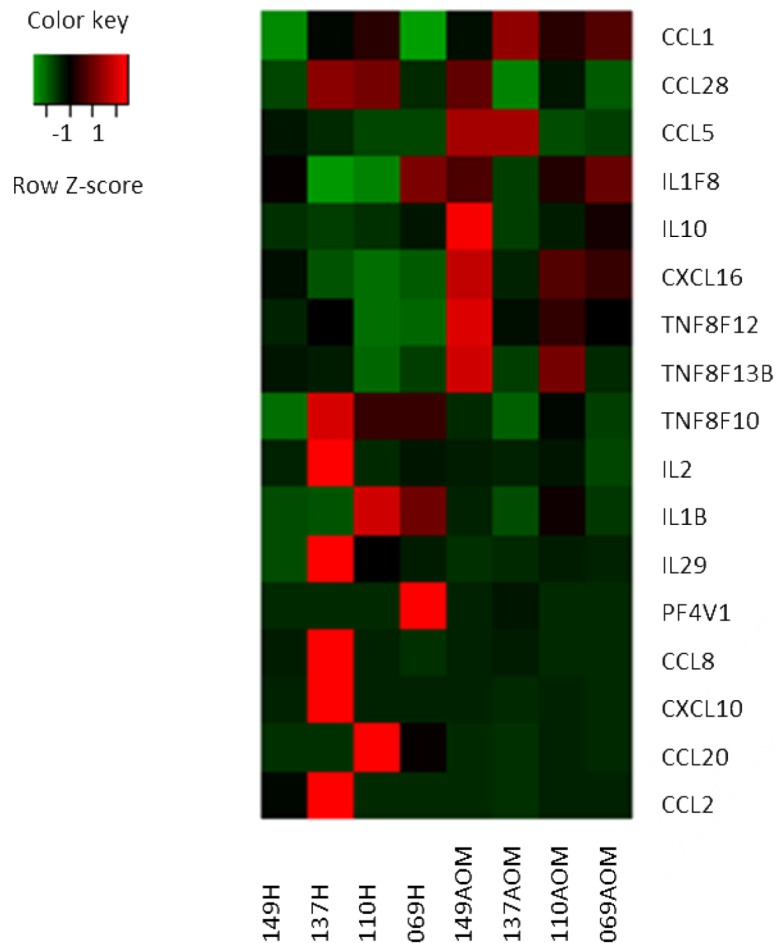


Fig. 3. Variability of gene expression among 4 children with AOM due to *Spn*
 Statistical package software R was used for the identification of differentially expressed genes. The profile includes 17 genes selected from the 127 immune defense response gene transcripts of PBMCs, which were >2 fold change at the comparison of the onset of AOM due to *Spn* and their pre-infection healthy stage. Shown are normalized expression (AOM stage) greater than (red), near (black), or less than (green) the mean of controls (healthy stage). Each column represents one child of healthy or AOM stage. Genes or transcripts are represented in rows.

Table 1
Comparison of the fraction of the genes differentially expressed in differential disease models

Spn-AOM: data derived from microarray in 4 children with AOM caused by *Spn* infection. Nontypeable *Haemophilus influenzae*

(*NTHi*)-AOM: data derived from microarray in 4 children with AOM caused by *NTHi*. *Spn* (mice): data derived from microarray in mice infected by *Spn*.

Disease model	Gene differentially expressed	Ratio of total probes (%)	Exp/Control (A/H)	Reference
<i>Spn</i> -AOM (Human)	1903	6.2	>2 fold	This study
<i>NTHi</i> -AOM (Human)	1487	4.8	>2 fold	Our Lab
HPV31 virus (Mouse)	7075	2	>2 fold	Chang, 2000
<i>Spn</i> (Mouse)	490	3.2	>1.5 fold	Zhang, 2006
THP-1, <i>Spn</i> (in vitro)	142	3.4	> 2 fold	Rogers, 2003
A549 cell, <i>P. aeruginosa</i> (in vitro)	22	1.5	>2 fold	Ichikawa, 2000

Table 2
Principal biological annotation and functional terms identified by gene set enrichment analysis

The genes differentially expressed in 4 children with AOM due to *Spn* relative to their pre-infection healthy stage were analyzed. Only of genes with a high fold change (< 0.5 and > 2 fold) in expression and enrichment score >1.3 and $P < 0.05$ occurring in all 4 children are shown.

Cluster* 1 Enrichment Score: 2.01				
Term**	Count	%	P Value	Fold Enrichment
GO:0016064, immunoglobulin mediated immune response	14	0.77	0.0019	2.62
GO:0019724, B cell mediated immunity	14	0.77	0.0027	2.52
GO:0002460, adaptive immune response based on somatic recombination of immune receptors	16	0.88	0.0078	2.10
GO:0002250, adaptive immune response	16	0.88	0.0078	2.10
GO:0002449, lymphocyte mediated immunity	15	0.82	0.0079	2.16
GO:0002455, humoral immune response mediated by circulating immunoglobulin	9	0.49	0.0090	2.93
GO:0006958, complement activation, classical pathway	8	0.44	0.0205	2.78
GO:0002252, immune effector process	21	1.15	0.0409	1.58
GO:0002443, leukocyte mediated immunity	15	0.82	0.0426	1.76
Total	128			
Cluster 2 Enrichment Score: 1.671				
Term	Count	%	P Value	Fold Enrichment
GO:0045935, positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	82	4.49	0.0064	1.33
GO:0051173, positive regulation of nitrogen compound metabolic process	84	4.60	0.0070	1.32
GO:0045941, positive regulation of transcription	74	4.05	0.0099	1.32
GO:0010557, positive regulation of macromolecule biosynthetic process	84	4.60	0.0102	1.30
GO:0031328, positive regulation of cellular biosynthetic process	87	4.76	0.0118	1.28
GO:0009891, positive regulation of biosynthetic process	88	4.82	0.0122	1.28
GO:0010628, positive regulation of gene expression	75	4.11	0.0136	1.30
GO:0045944, positive regulation of transcription from RNA polymerase II promoter	47	2.57	0.0635	1.28
Total	621			
Cluster 3 Enrichment Score: 1.54				
Term	Count	%	P Value	Fold Enrichment
GO:0000902, cell morphogenesis	51	2.79	0.0070	1.45
GO:0000904, cell morphogenesis involved in differentiation	37	2.03	0.0097	1.53
GO:0048667, cell morphogenesis involved in neuron differentiation	32	1.75	0.0146	1.54
GO:0032989, cellular component morphogenesis	54	2.96	0.0147	1.37
GO:0048858, cell projection morphogenesis	36	1.97	0.0171	1.48
GO:0032990, cell part morphogenesis	36	1.97	0.0308	1.42
GO:0030030, cell projection organization	48	2.63	0.0404	1.32
GO:0007409, axonogenesis	28	1.53	0.0415	1.46
Total	322			
Cluster 4 Enrichment Score: 1.31				

Cluster* 1 Enrichment Score: 2.01

Term**	Count	%	P Value	Fold Enrichment
Term	Count	%	P Value	Fold Enrichment
GO:0055074, calcium ion homeostasis	30	1.64	0.0106	1.61
GO:0055066, di-, tri-valent inorganic cation homeostasis	36	1.97	0.0120	1.52
GO:0006874, cellular calcium ion homeostasis	29	1.59	0.0132	1.60
GO:0030005, cellular di-, tri-valent inorganic cation homeostasis	34	1.86	0.0159	1.51
GO:0055065, metal ion homeostasis	31	1.70	0.0191	1.53
GO:0006875, cellular metal ion homeostasis	29	1.59	0.0304	1.49
Total	189			

* Number of functional annotation cluster (enrichment score >1.3).

** Number of functional annotation terms (enrichment of >1.3 and P < 0.051. These genes were analyzed using DAVID, software [<http://david.abcc.ncifcrf.gov>].

Table 3
Fold change of host immune defense response in children with AOM due to *Spn*

Genes are grouped functionally based on ontologies. RefSeq identifier: Entrez gene ID. The fold change of gene expression was derived by comparison of the gene expression intensity at the onset of AOM due to *Spn* with the pre-infection healthy stage of the same children. The data from the four children are presented as a mean.

Entrez gene ID	Gene Symbol	Gene description	Fold change
Genes associated with complement activation and TLR			
NM_134470	IL1RAP	interleukin 1 receptor accessory protein	6.69
NM_015991	C1QA	complement component 1, q subcomponent, A chain	6.33
NM_000491	C1QB	complement component 1, q subcomponent, B chain	4.46
NM_000877	IL1R1	interleukin 1 receptor, type I	4.35
NM_022059	CXCL16	chemokine (C-X-C motif) ligand 16	2.48
NM_003268	TLR5	toll-like receptor 5	2.31
NM_001928	CFD	complement factor D (adipsin)	2.25
NM_007268	VSIG4	V-set and immunoglobulin domain containing 4	2.23
NM_145343	APOL1	apolipoprotein L, 1	2.09
NM_183236	RAB27A	RAB27A, member RAS oncogene family	2.01
NM_001733	C1R	complement component 1, r subcomponent	-2
NM_000562	C8A	complement component 8, alpha polypeptide	-2.17
NM_000715	C4BPA	complement component 4 binding protein, alpha	-2.36
NM_005582	CD180	CD180 molecule	-2.48
NM_201442	C1S	complement component 1, s subcomponent	-2.6
NM_001145805	IRGM	immunity-related GTPase family, M	-3.13
NM_001877	CR2	complement component receptor 2	-3.32
NM_001032295	SERPING1	serpin peptidase inhibitor, member 1	-3.76
NM_001017388	TLR10	toll-like receptor 10	-3.97
Genes associated with antibacterial activity			
NM_153324	DEFB123	Beta-defensin 123	4.78
NM_005621	S100A12	S100 calcium-binding protein A12	4.52
NM_021058	HIST1H2BJ	Histone H2B type 1-J	3.59
NM_002965	S100A9	S100 calcium binding protein A9	2.71
NM_001725	BPI	Bactericidal permeability-increasing protein	2.59
NM_005091	PGLYRP1	Peptidoglycan recognition protein	-2.06
NM_207469	DEFB132	Beta-defensin 132	-3.07
NM_002343	LTF	Lactotransferrin	-3.98
Cytokine genes			
NM_002981	CCL1	chemokine (C-C motif) ligand 1	4.77
NM_148672	CCL28	chemokine (C-C motif) ligand 28	4.6
NM_002985	CCL5	chemokine (C-C motif) ligand 5	3.92
NM_014438	IL1F8	interleukin 1 family, member 8 (eta)	2.93
NM_000572	IL10	interleukin 10	2.93
NM_022059	CXCL16	chemokine (C-X-C motif) ligand 16	2.48

Entrez gene ID	Gene Symbol	Gene description	Fold change
NM_172088	TNFSF12, 13	tumor necrosis factor, member 12, 13	2.23
NM_006573	TNFSF13B	tumor necrosis factor superfamily, member 13b	2.17
NM_003810	TNFSF10	tumor necrosis factor superfamily, member 10	-2.05
NM_000586	IL2	interleukin 2	-2.12
NM_000576	IL1B	interleukin 1, beta	-2.38
NM_172140	IL29	interleukin 29 (interferon, lambda 1)	-3.17
NM_002620	PF4V1	platelet factor 4 variant 1	-6.18
NM_005623	CCL8	chemokine (C-C motif) ligand 8	-6.31
NM_001565	CXCL10	chemokine (C-X-C motif) ligand 10	-9.3
NM_004591	CCL20	chemokine (C-C motif) ligand 20	-9.77
NM_002982	CCL2	chemokine (C-C motif) ligand 2	-11.13
Genes for lymphocyte activation and differentiation			
NM_001765	CD1C	T-cell surface glycoprotein CD1c	-2.13
NM_001033667	LY9	T-lymphocyte surface antigen Ly-9	-2.16
NM_181780	BTLA	B- and T-lymphocyte attenuator	-2.34
NM_001039933	CD79B	B-cell Ag receptor complex-associated protein beta-chain	-2.39
NM_000073	CD3G	T-cell surface glycoprotein CD3 gamma chain	-2.49
NM_001783	CD79A	B-cell Ag receptor complex-associated protein alpha-chain	-2.53
NM_001770	CD19	B-lymphocyte antigen CD19	-2.81
NM_005191	CD80	T-lymphocyte activation antigen CD80	-3.69
NM_001025159	CD74	HLA class II histocompatibility antigen gamma chain	-3.39
NM_152866	CD20	membrane-spanning 4-domains, subfamily A	-3.41
NM_015055	SWAP70	SWAP switching B-cell complex 70kDa subunit	-2.64
NM_013314	BLNK	B-cell linker protein	-2.33
NM_000586.3	IL2	interleukin 2	-2.12
NM_032682	FOXP1	forkhead box P1	-2
NM_145912	NFAM1	NFAT activating protein with ITAM motif 1	2
NM_183236	RAB27A	RAB27A, member RAS oncogene family	2.01
NM_005178	BCL3	B-cell CLL/lymphoma 3	2.19
NM_007268	VSIG4	V-set and immunoglobulin domain containing 4	2.23
NM_000676	ADORA2B	Adenosine A2b receptor	2.53
NM_005442	EOMES	eomesodermin homolog	2.55
NM_002485	NBN	nibrin	2.8
NM_000572	IL10	interleukin 10	2.93