Downregulation of IL7R, CCR7, and TLR4 in the Cord Blood of Children With Respiratory Syncytial Virus Disease

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The association between gene expression at birth of 11 candidate genes with important innate and adaptive immune functions and later respiratory syncytial virus (RSV) disease was investigated. Cord blood was collected from 2108 newborns. Forty-seven were subsequently RSV positive. Gene expression analysis by quantitative reverse transcription–polymerase chain reaction was compared to 17 controls. There was downregulation of interleukin 7 receptor (IL7R) \( (P = .0001) \) and chemokine receptor 7 (CCR7) \( (P = .002) \), and in the severe disease subcategory, downregulation of Toll-like receptor 4 (TLR4) \( (P = .003) \). IL7R and CCR7 facilitate communication between adaptive and innate immune systems. TLR4 activates the innate immune system on RSV exposure. Delayed innate and adaptive immune activation may predispose children to more severe RSV disease.

Keywords. CCR7; children; dendritic cells; gene expression; IL7; IL7R; immunity; lymphocytes; respiratory syncytial virus; TLR4.

BACKGROUND

Respiratory syncytial virus (RSV) causes yearly epidemics of bronchiolitis and viral pneumonia in the pediatric population.

About two thirds of all children are exposed the first year of life, and almost all by the age of 2 years [1]. Most have mild upper airway disease. For every 1000 RSV cases, 22–31 required hospital admission [1, 2], but these children usually had a normal gestation and birth, and otherwise appeared healthy. Why so few exposed to RSV require hospital admission is a focus of on-going research.

Cellular responses to RSV are summarized in several reviews [3, 4]. Pulmonary dendrocytes are key innate immune cells of the respiratory epithelium that recognize RSV via Toll-like receptor 4 (TLR4). Dendrocytes then recruit granulocytes and monocytes, and migrate to regional lymph nodes, where they present RSV antigen to lymphocytes, mobilizing the adaptive immune system.

In this study, we hypothesize that differential regulation at birth of ligands and receptors involved in the immune response to RSV predisposes to later RSV disease, including proteins involved in the regulation of lung pathology by hyaluronan, chemotraction and chemotaxis of myeloid and lymphoid cells, interactions between cells of innate and adaptive immune systems, and regulation of lymphocyte responses.

METHODS

The Akershus Birth Cohort is described in our previous papers [5, 6]. From 3500 births, we collected 2108 cord blood samples into PAXgene collection tubes (PreAnalytiX GmbH, Hombrechtikon, Switzerland) and ethylenediaminetetraacetic acid (EDTA) tubes.

We cross-referenced the microbiology database with the birth cohort to identify participants who later had a positive RSV test. Patients were included if RSV positive before 36 months of age. Seventeen individuals not tested for RSV were randomly selected from the cohort as controls.

Exclusion Criteria

Cases and controls were assessed for the following exclusion criteria September 2009, at age 5–6 years: (1) conditions known to predispose to severe RSV disease: congenital heart disease, gestational age at birth <34 weeks; chronic airways diseases (eg, bronchopulmonary dysplasia, asthma, recurrent lower airway infections, congenital airway anomalies), or Down’s syndrome. (2) Conditions affecting gene regulation at birth: small for gestational age, perinatal infection, metabolic or neurological disease. (3) Controls who had moved from our hospital population area, or who were admitted to hospital with other respiratory disease.
**Disease Categorization**
Medical records were assessed retrospectively. Criteria for severe RSV disease were mechanical respiratory support needed, had apnea related to respiratory exhaustion, supplementary oxygen required, intravenous or nasogastric fluid needed, or had significant level of respiratory distress, as documented by the pediatrician. Criteria for mild RSV disease were that the patient was tested for RSV by their general practitioner (GP) but not sent to hospital for admission, sent home from hospital without admission, had only upper-airways disease, or had mild or no respiratory distress, as assessed by the pediatrician.

**Quantitative Reverse Transcription–Polymerase Chain Reaction Analysis**
Total RNA was isolated from cord blood samples and prepared as described in our previous paper [5]. Gene expression was quantified by quantitative reverse transcription–polymerase chain reaction (RT-qPCR) of mRNA for IL7RA, CCR7, CXCL7, TLR4, IL4, IFNG, CCL3, CXCL11, CXCR3, CD44, and RHAMM. Please see the Supplementary Material for a detailed description of the RT-qPCR analysis.

**Protein Analysis**
Genes significantly regulated in the RT-qPCR were selected for protein analysis. Because these genes encode cell membrane-bound proteins, EDTA samples were analyzed. Ten samples from each group (control, mild, and severe disease), representative of the interquartile range for mRNA expression for each target protein, were analyzed. Protein extraction and Western blot analysis were carried out as described previously [5].

In our 2003 study, cell-layer separation resulted in contamination with erythrocytes and plasma in our leukocyte samples. To correct for this, target proteins were normalized against the leukocyte-specific surface protein CD45 in our samples [7]. To verify leukocyte specificity, new cord blood samples were collected in 2011, and leukocyte, erythrocyte, and plasma layers were carefully separated and analyzed by Western blotting. Please see the Supplementary Material for a detailed description of the protein analysis.

**Statistical Analysis**
RT-qPCR data were assessed for normal distribution using the Kolmogorov-Smirnov and Shapiro-Wilk tests. Normally and nonnormally distributed data were analyzed using Student t test and the Mann–Whitney test, respectively. The difference in medians was calculated using the Hodges–Lehmann estimator. Differences in mean or median were first calculated for calibrated and normalized cycle threshold (ΔΔCt) values, and then converted to relative quantities. Subgroup analysis for severity of disease utilized 1-way ANOVA or the Kruskal–Wallis test. For each sample, the target-protein-to-CD45 ratio was calculated, log transformed, and analyzed using Student t test. A power analysis of previous results indicated a need for 11 individuals in each group for a power of 80% in the mRNA analysis. We included 17 in the control group. Statistical analyses were carried out using Prism 5 (GraphPad Software Inc), SPSS 19, and Minitab 15.0 statistical software. Using the Bonferroni correction, $P < .0045$ was considered significant.

**Ethical Issues**
Regional Ethics Committee approval was obtained before data collection began. A guardian gave informed, written consent before study inclusion.

**RESULTS**
Of 2108 children, 70 were RSV positive before age 3 years; 3 had asthma, 1 had repeated pneumonia, 1 had cleft palate, and 1 had Down’s syndrome. Sixty-four were eligible for inclusion; 47 had sufficient RNA for analysis. The mean age on positive RSV testing was 7.7 months (SD, 9.4 months); median age 3 months (interquartile range, 2–8 months); 37 were <12 months of age and 10 were 12–35 months of age. Twenty-five had severe and 22 had mild disease. Birth, clinical, and intervention data are presented in Table 1. Eight RSV-positive patients were not referred to hospital and were therefore classified with mild disease.

**Gene Expression**
When considering all children with confirmed RSV infection, there was a significant downregulation of both IL7R (relative expression 69%; 95% confidence interval [CI], 58%–83%; $P = .0001$) and CCR7 (relative expression 78%; 95% CI, 67%–91%; $P = .002$) in the RT-qPCR analysis (Figure 1A). On subgroup analysis for disease severity (Figure 1B), IL7R and CCR7 had a significant 1-way ANOVA ($P = .0006$ and $P = .004$, respectively), and TLR4 tended to significance ($P = .02$). Post-hoc analysis revealed significant downregulation of TLR4 among children with severe RSV disease compared to controls (relative expression 64%; 95% CI, 48%–85%; $P = .003$).

**Protein Analysis**
Western blot analysis to verify CD45 specificity is presented in the Supplementary Material (Supplementary Figure 1). We can correct for erythrocyte but not plasma contamination, due to nonspecific anti-CD45 antibody binding to plasma proteins. Plasma contamination is likely to be evenly distributed between groups, reducing leukocyte-protein concentration, but not the ratio of means. However, intragroup variation is likely, reducing statistical power.

IL7R, CCR7, and TLR4 were selected for protein analysis. Figure 1C presents CD45 ratios for controls and cases. There were no significant associations but there are obvious trends that support our mRNA findings.
DISCUSSION

In this nested case-control study, we found downregulation of IL7R and CCR7 at birth in the cord blood of children who are later RSV positive. There was significant downregulation of TLR4 in patients later admitted with severe disease. For several genes, a large variance meant a lack of power to detect clinically interesting differences. This was particularly relevant for CXCR3, IFNG, IL4, RHAMM, and CXCL11.

While protein results do not statistically confirm our mRNA findings, the trends do support them. The Western blot experiment may have lacked power to detect significant differences. We cannot exclude altered protein expression due to posttranscriptional regulation of mRNA by micro-RNA or other mechanisms.

Control individuals were probably exposed to RSV. We have no information about their clinical symptoms other than that they did not present to pediatric units in our region, and that their GP did not send a nasopharyngeal aspirate for RSV.

<table>
<thead>
<tr>
<th>Table 1. Birth, Clinical, and Intervention Data for Control, Mild RSV, and Severe RSV Groups</th>
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<tbody>
<tr>
<td>Control</td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td>Number</td>
</tr>
<tr>
<td>Male : female ratio</td>
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<tr>
<td>Birth data: (n = 17)</td>
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<tr>
<td>Vaginal</td>
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<tr>
<td>Instrumental</td>
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<tr>
<td>Caesarian section</td>
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<tr>
<td>Mean gestational age (SD), weeks</td>
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<tr>
<td>Mean birth weight (SD), grams</td>
</tr>
<tr>
<td>1-minute Apgar &lt;8</td>
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<tr>
<td>5-minute Apgar &lt;8</td>
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<tr>
<td>Clinical features: (n = 14)</td>
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<tr>
<td>Median age RSV positive (IQR), months</td>
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<tr>
<td>Significant dyspnea (^d )</td>
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<tr>
<td>Apnea</td>
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<td>Cardiovascular compromise</td>
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<td>Highest pCO2, mean (SD), kPa</td>
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<td>Lowest O2 saturation; mean (SD), %</td>
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<tr>
<td>Highest respiratory rate; mean (SD), /minutes</td>
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<td>Pulse/min on admission; mean (SD)</td>
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<td>Weight on admission; mean (SD), grams</td>
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<tr>
<td>Duration of symptoms on admission; mean (SD), days</td>
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<td>Length of stay; median (IQR), days</td>
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<tr>
<td>Interventions:</td>
</tr>
<tr>
<td>CPAP/ventilator</td>
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<tr>
<td>Supplemental oxygen</td>
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<td>Supplemental fluids:</td>
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<td>Intravenous</td>
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There were no significant differences in gestational or delivery factors that might affect gene expression. Patients in mild and severe groups were ill at similar ages. There was a statistically significant reduction in oxygen saturation, increase in respiratory rate, and longer length of stay in the severe disease group, and a tendency to higher pCO2. Clinical features and interventions incorporated in the algorithm for disease severity were not analyzed statistically.

Abbreviations: ANOVA, analysis of variance; CPAP, continuous positive airway pressure; IQR, interquartile range; pCO2, capillary partial pressure of carbon dioxide; RSV, respiratory syncytial virus.

\( a \) \( \chi^2 \) test comparing all 3 groups.
\( b \) Birth records were missing for 4 patients with mild and 1 patient with severe disease.
\( c \) 1-way ANOVA.
\( d \) Apart from age on RSV positivity and gender, clinical features were unavailable for 8 RSV-positive patients not referred to hospital, classified with mild disease.
\( e \) Mann–Whitney test.
\( f \) Pediatrician’s assessment of respiratory effort.
\( g \) Lowest O2 saturation irrespective of oxygen administration.
\( h \) Student t test.
tendency for GPs in our area to refer younger children for assessment by a pediatrician, we believe it probable that control individuals had a mild course of RSV disease (such as rhinitis), and that the chance of severe RSV disease in the control group was low. However, we cannot exclude overlap between mild and control groups. Disease severity subgroup allocation may be confounded by retrospective collection of clinical data, leading to erroneous classification. This will not have affected the results for IL7R or CCR7, where mild and severe groups were similar, but may affect results for TLR4.

IL7RA is in peripheral blood expressed by mature T-lymphocytes, closely determining cellular responses to interleukin-7 (IL7). IL7 signaling is an important regulator of T-cell survival and homeostasis, preventing apoptosis, promoting T-cell proliferation in some conditions, maintaining peripheral T-lymphocyte levels during immune responses, and possibly regulating effects of T-cell receptor stimulation. IL7- and IL7-RA-deficient mice have depleted thymocyte and T-lymphocyte levels [8].

CCR7 regulates dendrocyte and lymphocyte migration, both to and within lymph nodes. This migration is essential for communication between innate and adaptive immune systems. CCR7 is therefore required for the efficient induction and regulation of adaptive immune responses. CCR7 is also involved in the development of antigen tolerance [9].

TLR4 is a pathogen recognition receptor of the innate immune system, expressed on pulmonary epithelial cells, and in abundance on pulmonary dendrocytes. TLR4 single-nucleotide polymorphisms increase the risk of RSV disease [10]. Binding of RSV antigen to TLR4 initiates innate immune responses involving nuclear factor \( \kappa B \) (NF-\( \kappa B \))-mediated production of signaling and antiviral factors; dendrocyte activation; granulocyte, macrophage, and natural killer cell recruitment; and dendrocyte migration to lymph nodes, where they initiate the adaptive immune response [4, 11]. TLR4 is also a low-molecular-weight hyaluronan receptor [12]. CD44 mediates immune responses initiated by hyaluronan/TLR4 interactions [13], but its expression was not associated with predisposition to RSV infection.

IL7RA and CCR7 are not previously associated with RSV, but mice deficient in these proteins have a severe combined immunodeficiency phenotype [8, 9]. The clinical relevance of gene expression levels seen in this study is unknown. The children in our study did not have life-threatening disease, chronic disorders, or repeat infections. The immune phenotype predisposing to RSV disease that is suggested by this study is therefore not likely to lead to severe immunodeficiency.

Cohort samples were taken after a stressful experience (birth), evoking an immune response, and we can therefore make conclusions on the immune response of these neonates. We previously reported downregulation of tumor necrosis factor receptor 25, Dicer, and the NF-\( \kappa B \) system in this cohort.
In light of these and current findings, it seems reasonable to speculate that in children who develop RSV disease, immunological differences present at birth may involve initiation of the innate immune response, communication between innate and adaptive immune systems, and homeostasis of peripheral T lymphocytes. A unifying cause explaining all of our findings might be genetic or epigenetic variations in 1 or more of the genes we have described. Intergroup variation in the differential leukocyte count could also explain our findings, particularly for IL7R, which is likely expressed by lymphocytes; and TLR4, which is likely expressed by myeloid lineages. Unfortunately, we did not perform such a count in our cohort. A recent study has described no differences in innate immune cell count at 1 month of age between infants with and infants without later RSV infection [14].

El Saleby et al [15] described a greater risk of severe disease in children with higher RSV nasal viral loads and delayed viral clearance. Our findings may explain this association: an impaired immune response due to reduced activation of the innate immune response, and delayed communication between innate and adaptive immune systems could allow for greater viral replication, more extensive epithelial cell infection, and a greater risk of pulmonary dissemination early during the course of infection, ultimately leading to greater RSV-mediated cell death, and therefore a more severe immune response when cells of the innate and adaptive immune systems have been adequately recruited.

CONCLUSION

We found downregulation of IL7R and CCR7 in the cord blood of children later testing positive for respiratory syncytial virus. In addition, those with severe disease had significant downregulation of TLR4. Downregulation of these leukocyte cell-surface receptors may cause an impaired immune response to RSV, allowing greater viral replication and tissue damage, and thus more severe disease.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

References