Host immune response in children with Down syndrome related to respiratory tract infection

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Chapter 3

Increased production of interleukin-10 in children with Down syndrome upon ex-vivo stimulation with *S. pneumoniae*

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Abstract

Background: Children with Down syndrome (DS) have an increased susceptibility to infections, due to altered humoral and/or cellular immunity. The aim of the study was to determine the cytokine production in whole blood of children with DS upon stimulation with heat-killed *Streptococcus pneumoniae* and lipopolysaccharide (LPS), in comparison with their healthy siblings.

Methods: Whole blood of 61 children with DS and 57 of their healthy siblings was stimulated with 200 ng/ml LPS and 4 x 10^7 Colony Forming Units (CFU)/ml *S. pneumoniae* during 6, 24 and 48 hours. Concentrations of pro- and anti-inflammatory cytokines Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-1β, IL-6, IL-8, IL-12p70 and IL-10 were determined at all time points.

Results: Children with DS show an increased IL-10 production upon stimulation with *S. pneumoniae*, compared to their healthy siblings. At most time points no significant differences were seen in cytokine production upon stimulation with LPS.

Conclusion: Children with DS may be prone to a severe course of pneumococcal pneumonia, because of an increased anti-inflammatory response.

Introduction

Down syndrome (DS), trisomy 21, is one of the most common chromosomal disorders with a prevalence of 10 to 14 per 10,000 live births in the Netherlands[1] and a prevalence of 10.3 per 10,000 children in the USA.[2] Apart from mental retardation, children with DS have an increased incidence of congenital defects (heart and gastrointestinal tract), autoimmune disease (celiac disease) and malignancies (leukemia). Because of their predisposition to these medical conditions they need multidisciplinary medical care.[3,4] They are also more prone to respiratory tract infections (RTIs) which commonly manifest in the lower airways, a major cause of hospitalization.[5,6] Several factors contribute to increased risk of RTI in children with DS: neurological impairment,[7] abnormal anatomy of the upper airways,[8] structural pulmonary abnormalities[9] and congenital heart defects.[10] In addition, alterations in the immune system are an important cause of RTIs in DS children.[11,12] Defects in both the innate and the adaptive immunity are reported in DS, for example mannan-binding lectin deficiency,[13] a high number of pro-inflammatory $CD14^{dim}CD16^{+}$ monocytes,[14] changes in T and B lymphocyte counts,[15-17] early aging of the immune system,[18,19] an intrinsic defect of T and B lymphocytes,[16,20,21] IgG2 and IgG4 subclass deficiencies,[16,17,21-24] impaired antibody response to pneumococcal vaccine,[25] diminished invariant natural killer T cells[14,17] and regulatory T cells.[17] These lower RTIs in DS children are most often caused by viral pathogens, such as respiratory syncytial virus (RSV). This can lead to severe RSV bronchiolitis, a frequent cause of hospitalization in DS children.[10,26-28] Also an increased risk of hospitalization, endotracheal intubation and death due to influenza A virus infection was reported in DS.[29] In addition we found an increased pro-inflammatory cytokine response to live influenza A virus in children with DS, which might contribute to an increased severity of their clinical course of this infection.[30] Bacterial pathogens, both Gram positive and Gram negative can also cause lower RTIs in children. However, nothing is known about the immune response to these type of RTIs in children with DS. For this reason we used ex-vivo stimulation with *S. pneumoniae* and lipopolysaccharide (LPS) in whole blood of DS children and their healthy siblings as a model for a Gram positive and Gram negative bacterial RTI and we evaluated in the culture supernatants the levels of inflammatory mediators Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-1β, IL-6, IL-8 ,IL-12p70 and IL-10.

Patients and methods

Patients

The study was performed in the Vrije Universiteit Medical Center (VUmc) in Amsterdam, the Netherlands. The study protocol was approved by the Medical Ethics Committee of the VUmc. We invited 210 DS children from our Down syndrome outpatient clinic and their healthy siblings as controls to participate in the study. Inclusion criteria for children with DS were the following: DS diagnosis confirmed by chromosome analysis, age older than 3 months, no symptoms of infection at the time the blood sample was taken. Inclusion criteria for siblings (if present): age older than 3 months, no symptoms of infection at the time the blood sample was taken. The age older than 3 months was chosen because of possible technical difficulties to obtain enough blood for this study in very young children. Within one family the age of the sibling was matched as much as possible with the age of the child with DS. The parents of participating children gave their written informed consent. From each child 6 ml of heparinized blood was obtained by venipuncture. The blood samples were kept on ice while transporting to the laboratory.

Whole blood stimulation with *S. pneumoniae*

Heat-killed *S. pneumoniae* (ATCC6303) was diluted in RPMI 1640 supplemented with glutamine (0.5 mM) to a stock concentration of 4 x 10^7 Colony Forming Units (CFU)/ml. For each time-point, 250 µl of whole blood was incubated with 250 µl of the *S. pneumoniae* stock solution at 37°C and 5% CO_2; therefore the concentration of stimulation was 4 x 10^7 CFU/ml whole blood.

Whole blood stimulation with LPS

LPS (from *Escherichia coli* O55:B5; Sigma-Aldrich, St. Louis, Missouri) was diluted in RPMI 1640 supplemented with glutamine (0.5 mM) to a stock concentration of 200 ng/ml. For each time-point, 250 µl of whole blood was incubated with 250 µl of the LPS stock solution at 37°C and 5% CO_2; therefore the concentration of stimulation was 200 ng per ml whole blood. The remaining whole blood at t=0 and the blood at 6, 24 and 48 hours of incubation was centrifuged (48R centrifuge Hettich Rotina, Tuttlingen, Germany) for 10 minutes at 3000 rpm at 4°C and the supernatant was stored at -80°C until cytokine assays were performed.

Measurement of plasma inflammatory mediators

TNF-α, IL-1β, IL-6, IL-8, IL-10 were measured by Cytometric Bead Array (Human Inflammation Kit, BD CBA, BD Biosciences, San Diego, California) and IL-12p70 was measured by ELISA (Human IL-12(p70) Kit, BD OptEIA, BD Biosciences, San Jose California) in accordance with the manufacturer's recommendations.

Statistical analysis

The categoric variables were analyzed by the χ^2 test. Cytokine data were analyzed by the Mann-Whitney U test. Data are expressed as means ± standard error of the mean (SEM). A p-value of <0.05 was considered statistically significant.

Results

Patients and controls

After parental consent, 61 children with DS and 57 of their age matched healthy siblings were included in the study. In 8 families the child with DS was the only one to participate because there were no siblings. In 48 families 1 sibling per child with DS participated. In 5 families 2 siblings per child with DS participated. The average age (± standard deviation) in the DS group was 7.8 (±5.1) vs. 9.3 (± 5.5) years in the sibling group (p=0.13). A significant difference according to sex was found between both groups (39/61 (64%) male DS children vs. 23/57(40%) male siblings (p=0.02)).Chromosome analysis in the DS group revealed 1 child with a translocation of chromosome 21 and 60 children with trisomy 21.

Levels of inflammatory mediators

In Figure 3.1 the levels of TNF-α , IL-1β, IL-6, IL-8, IL-10 and IL-12p70 in DS children and their healthy siblings upon stimulation with *S. pneumoniae* at 0, 6, 24, and 48 hours are presented. IL-6 levels at 6 hours were significantly higher in the DS group. IL-10 levels were significantly higher in the DS group than in the sibling group after 24 hours and after 48 hours. IL-12p70 levels at 6 hours were significantly lower in the DS group. In Figure 3.2 the levels of TNF-α , IL-1β, IL-6, IL-8, IL-10 and IL-12p70 in DS children and their healthy siblings upon stimulation with LPS at 0, 6, 24, and 48 hours are presented. IL-1β levels at 48 hours are significantly lower in the DS group. No significant differences in IL-10 levels were seen between the two groups.

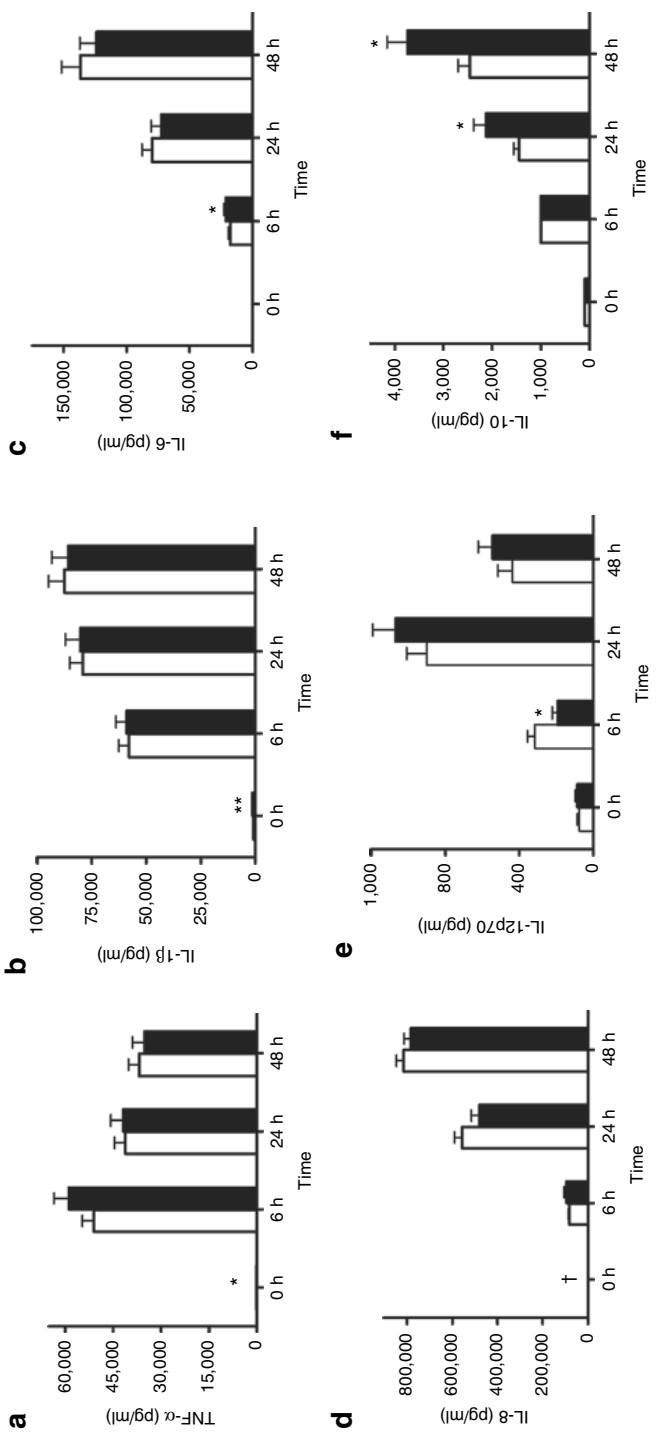

Figure 3.1A-F Cytokine levels, mean ± SEM, of Down syndrome children (black bars) and controls (white bars) after stimulation with heat-killed *S. pneumoniae*. [A] TNF-α levels. [B] IL-1β levels. [C] IL-6 levels. [D] IL-8 levels. [E] IL-12p70 levels. [F] IL-10 levels.
* $p<0.05$, ** $p<0.01$, † $p<0.001$.

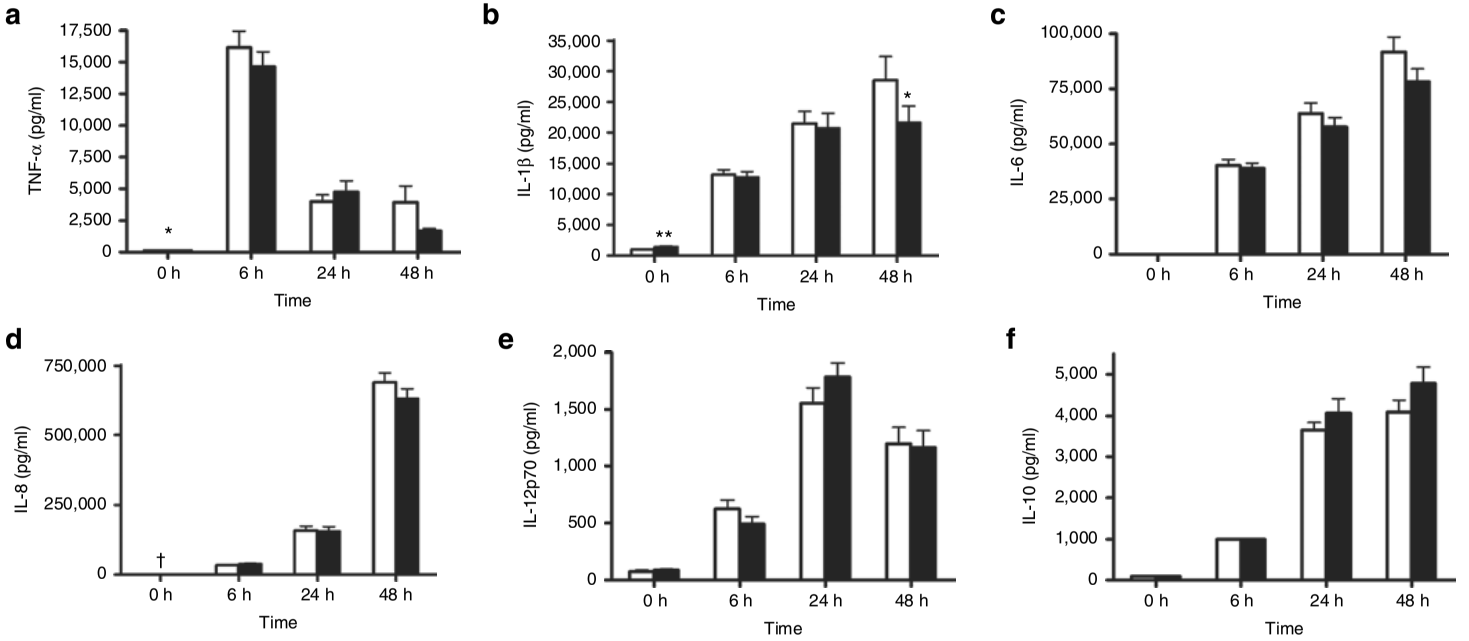

Figure 3.2A-F Cytokine levels, mean ± SEM, of Down syndrome children (black bars) and controls (white bars) after stimulation with LPS. [A] TNF-α levels. [B] IL-1β levels. [C] IL-6 levels. [D] IL-8 levels. [E] IL-12p70 levels. [F] IL-10 levels.

* p<0.05, ** p<0.01, † p<0.001.

Discussion

In the human respiratory tract, a continuous exposure to microorganisms is present. The first line of defence against these pathogens, which is part of the innate immune response, is mainly formed by the ciliated epithelium, dendritic cells and macrophages that are present locally. By phagocytosis and killing, the causing pathogen is destroyed and removed and thereby further tissue invasion is prevented. Also, a more specific immune response is initiated locally with the production of pro- and anti-inflammatory cytokines.[31] *S. pneumoniae* is an example of a Gram positive bacteria and a frequent cause of community acquired RTI.[32] Young children are at risk for invasive pneumococcal disease because of the immaturity of their immune system. We wanted to investigate whether DS children can be considered as an additional risk group for pneumococcal RTI. Because we wanted to unravel the underlying innate immune response to a Gram positive bacterial stimulus, we performed ex-vivo whole blood stimulation with *S. pneumoniae* in children with DS and their healthy siblings as a model for Gram positive bacterial pneumonia. The most important finding of our study is that children with DS produce increased levels of IL-10 upon ex-vivo stimulation with heat-killed *S. pneumoniae*. Many animal studies, especially in mice, have been performed investigating pulmonary *S. pneumoniae* infection. In mice with pneumonia induced by intranasal inoculation with *S. pneumoniae*, higher levels of the anti-inflammatory cytokine IL-10 were associated with decreased lung levels of TNF-α and IFN-γ, increased bacterial counts in lungs and blood and early lethality.[33] In our study IL-10 levels increased significantly from 6 to 48 hours in the DS group in comparison to the controls. In adults with pneumococcal pneumonia, high levels of IL-10 were present in serum at admission and declined within 48 hours while treated with antibiotics.[34] In another study in humans with pneumococcal pneumoniae, high levels of IL-10 increased the in-hospital mortality rate.[35] Hence, the elevated IL-10 levels we found in DS might be associated with a more severe course of *S. pneumoniae* pneumonia in DS but can be downregulated by the treatment with antibiotics. In the present study we also found significantly higher IL-6 levels at 6 hours in the DS group upon stimulation with *S. pneumoniae*. Van der Poll et al.[33] reported a protective effect of lung and plasma IL-6 in mice pneumonia after intranasal infection with *S. pneumoniae*; their mortality rate was less and the amount of bacteria in the lungs was less than in IL-6 knockout mice. In addition higher levels of pro-, and anti-inflammatory cytokines were present in the lung of the IL-6 knockout mice. Hence, IL-6 downregulates the activation of the cytokine network in the lung both controlling the activation of both agonist and antagonist mediators during pneumococcal pneumonia and thus contributes

to host defense.[36] However, in humans with pneumococcal pneumonia high levels of IL-6 in serum were associated with a more frequent admission to the intensive care unit and also a higher mortality.[37,38] In complicated pneumococcal pneumonia with pleural effusion in children, increased release of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 in pleural fluid resulted in increased complications with the formation of fibrin deposition requiring surgical intervention.[35,39] In our study we didn't find any differences in TNF-α and IL-1β in serum between DS children and the control group. However, IL-6 levels at 6 hours were significantly elevated in these children. Since no studies have been performed in DS children with pneumococcal pneumonia which measure local cytokine production in the lung, it is difficult to extrapolate our results to the clinical perspective of this specific group of patients. On the one hand, the elevated IL-6 levels we found in an early phase in DS children may protect against a severe clinical course of RTI when we have the mice experiments in mind; on the other hand, concerning the human studies as mentioned above, elevated IL-6 levels might have a deplorable effect.

In our study we performed ex-vivo whole blood stimulation with LPS, a very important virulence factor of gramnegative bacteria, in children with DS and their healthy siblings as a model for Gram negative bacterial pneumonia. In a Gram negative pneumonia model in mice, Herold et al. showed that acute lung injury was mediated by IL-1β and was attenuated by an IL-1 receptor antagonist.[40] In humans with Gram negative nosocomial pneumonia, elevated concentrations of TNF-α and IL-6 were present in blood, but IL-1β was undetectable.[41] High levels of IL-1β in bronchoalveloar lavage fluid of mechanically ventilated humans with a community-acquired pneumonia due to *Pseudomonas aeruginosa*, were associated with a high bacterial load in the alveoli. This was also associated with progressive inflammation of the lung.[42] Thus, the significantly lower levels of IL-1β at 48 hours we found in children with DS might protect them from acute lung injury in Gram negative pneumonia. No significant differences in IL-10 levels were seen in these mice, as we found upon stimulation with *S. pneumoniae*.

Our results demonstrate that different micro-organisms play an important role in the host response and trigger different inflammatory responses, depending on their intrinsic properties.Pneumococci and LPS both interact with TLR4 on innate immune cells, but in addition pneumococci also interact with TLR1 and TLR2 on innate immune cells which possibly leads to the difference in IL-10 production between the LPS and *S. pneumoniae* stimulations.[43] The strength of our study is that, by choosing their age-matched siblings as a control group for the DS children, we minimized genetic, environmental and age-

related differences.There is also a limitation of our study: we have measured the systemic inflammatory response by measuring cytokine levels in the blood, which might not correlate with the cytokine levels in the pulmonary compartment during pneumonia. However, Kragsbjerg et al. demonstrated high circulating levels of IL-8 in patients with community-acquired pneumonia caused by *S. pneumoniae*[44] and Bonten et al. showed that high circulating levels of IL-6 and IL-8 were associated with higher mortality rates.[45] Further studies are necessary to address this.

Conclusion

Children with DS show an increased IL-10 production in response to ex-vivo stimulation with *S. pneumoniae*. This might result in a more severe course of pneumococcal disease in children with DS.

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