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

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Chapter 4

Increased pro-inflammatory cytokine production in Down syndrome children upon stimulation with live influenza A virus

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Abstract

Purpose: Children with Down Syndrome (DS) have an increased susceptibility to infections, due to altered humoral and/or cellular immunity. The aim of this study was to determine the cytokine production in whole blood of children with DS upon stimulation with live influenza A virus.

Methods: Whole blood of 61 children with DS and 57 of their healthy siblings was stimulated with 2.5 x 10^4 $TCID_{50}$/ml influenza A virus during 6, 24 and 48 hours. TNF-α, IL-1β, IL-6, IL-8, IL-10, IL-12p70, IFN-α, IFN-γ concentrations and viral load were measured at all time points.

Results: At most of the time-points TNF-α, IL-1β, IL-6 and IL-8 concentrations were significantly higher in children with DS following stimulation with live influenza A virus. IFN-α and IFN-γ levels were also significantly higher in the DS group. Viral clearance however, was equal in both groups.

Conclusions: Children with DS have an altered immune response to influenza A virus. The production of higher levels of pro-inflammatory cytokines may be responsible for a more severe clinical course of viral disease in these children.

Introduction

Down syndrome (DS), trisomy 21, is one of the most common chromosomal disorders with an incidence of 1 in 625 in the Netherlands[1] and a prevalence of 10.3 per 10,000 children in the USA.[2] Children with DS have an increased incidence of congenital malformations of the heart and the gastrointestinal tract, malignancies (e.g. leukemia) and autoimmune disease (e.g.celiac disease), apart from mental retardation. Because of these potential medical risks, children with DS need multidisciplinary care and follow up.[3,4]

Children with DS also have a higher risk of respiratory tract infections (RTIs) which commonly manifest in the lower airways.[5,6] These lower RTIs are most often caused by viral pathogens, such as respiratory syncytial virus (RSV). This can lead to severe RSV bronchiolitis which in its turn is a frequent cause of hospitalization in DS children.[7-10] Very recently it was shown that children with DS also have an increased risk of hospitalization, endotracheal intubation and death due to influenza A virus infection.[11]

The development of these RTIs may be affected by several different factors, like neurological impairment,[12] abnormal anatomy of the upper airways,[13] structural pulmonary abnormalities[14] and congenital heart defects.[8] In addition, changes in the immune system play a special role as potential cause of RTIs in DS children, which we have recently published in a review.[15] Both defects in the innate and the adaptive immunity, for example mannan-binding lectin deficiency,[16] a high number of pro-inflammatory $CD14^{dim}CD16^{+}$ monocytes,[17] changes in T and B lymphocyte counts,[18,19] early aging of the immune system,[20,21] an intrinsic defect of T and B lymphocytes[19,22,23] and IgG2 and IgG4 subclass deficiencies[19,23-26] have been reported. We hypothesize that one of the reasons that children with DS have a more severe course of viral infections is an altered innate immune response consisting of different cytokine responses. To study this, we used ex-vivo stimulation with live influenza A virus in whole blood of DS children as a model for viral respiratory tract infection and evaluated plasma levels of inflammatory mediators (TNF-α, IL-1β, IL-6, IL-8 and IL-12p70, IL-10, INF-α, INF-γ).

Patients and methods

Patients

The study was performed in the VU University 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: DS diagnosis confirmed by chromosome analysis, age older than 3 months, no symptoms of infection (by medical history, physical examination, and laboratory examination: C reactive protein and leukocyte count) at the time the blood sample was taken. Inclusion criteria for siblings (if present): age older than 3 months, no symptoms of infection (by medical history, physical examination, and laboratory test: C reactive protein and leukocyte count) 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.

Whole blood stimulation with influenza A virus

Influenza A/PR/8/34 (VR-95; ATCC, Rockville, MD, USA) was grown on LLC-MK2 cells (RIVM, Bilthoven, the Netherlands) and isolated as previously described.[27] The viral stock was diluted in RPMI 1640 supplemented with glutamine (0.5 mM) and 0.1% fetal calf serum to a final dose of 2.5 x 10^4 median tissue culture infective dose ($TCID_{50}$)/ml. From each child 6 ml of heparin blood was obtained by venapuncture. The blood samples were kept on ice while transported to the laboratory. For each time-point, 250 µl of whole blood was incubated with 250 µl of the 2.5 x 10^4 $TCID_{50}$/ml live influenza A virus at 37°C and 5% CO_2. The remaining whole blood was centrifuged (Hettich Rotina 48R centrifuge) for 10 minutes at 3000 rpm at 4°C and the serum was stored at -80°C (t=0). At 6, 24 and 48 hrs incubation the blood was centrifuged for 10 minutes at 1200 rpm at 4°C and the supernatant was stored at -80°C until cytokine assays were performed.

Measurements of viral RNA copies: at 6, 24 and 48 hrs 10 ml phosphate-buffered saline (PBS) was added to the remaining cells and centrifuged for 3 minutes at 1200 rpm at 4°C. The supernatant was removed, subsequently 10 ml NaCl 0.9% was added and centrifuged for 3 minutes at 1200 rpm at 4°C. The supernatant was removed and 3 ml red blood cell lysis buffer (ammonium chloride) was added and incubated on ice for a maximum of 10 minutes. Subsequently 7 ml of PBS was added and centrifuged for 3 minutes at 1200 rpm at 4°C. This step was repeated in the case of insufficient red blood cell lysis. After adequate red blood cell lysis, 1 ml of Trizol was added to the remaining cell pellet and stored at -20°C

until viral load assays were performed (within one month after isolation). Measurements of viral RNA copies were performed by Taqman real-time quantitative PCR as described.[27]

Measurement of plasma inflammatory mediators

TNF-α, IL-1β, IL-6, IL-8, IL-10 and IL-12p70 were measured by Cytometric Bead Assay (Human Inflammation Kit, BD™CBA, BD Biosciences, San Diego, CA, USA) in accordance with the manufacturer's recommendations. IFN-α (Human IFNα, module set, Bender MedSystems GmbH ,Vienna, Austria) and IFN-γ (Human IFNγ, DuoSet, R&D Systems Europe, Ltd, Abingdon, United Kingdom) were measured by ELISA in accordance with the manufacturer's recommendations.

Statistical analysis

The categoric variables were analyzed by the χ^2 test. Cytokine and interferon 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 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=1.00). 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 4.1 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 live influenza A virus at 6, 24, and 48 hours are

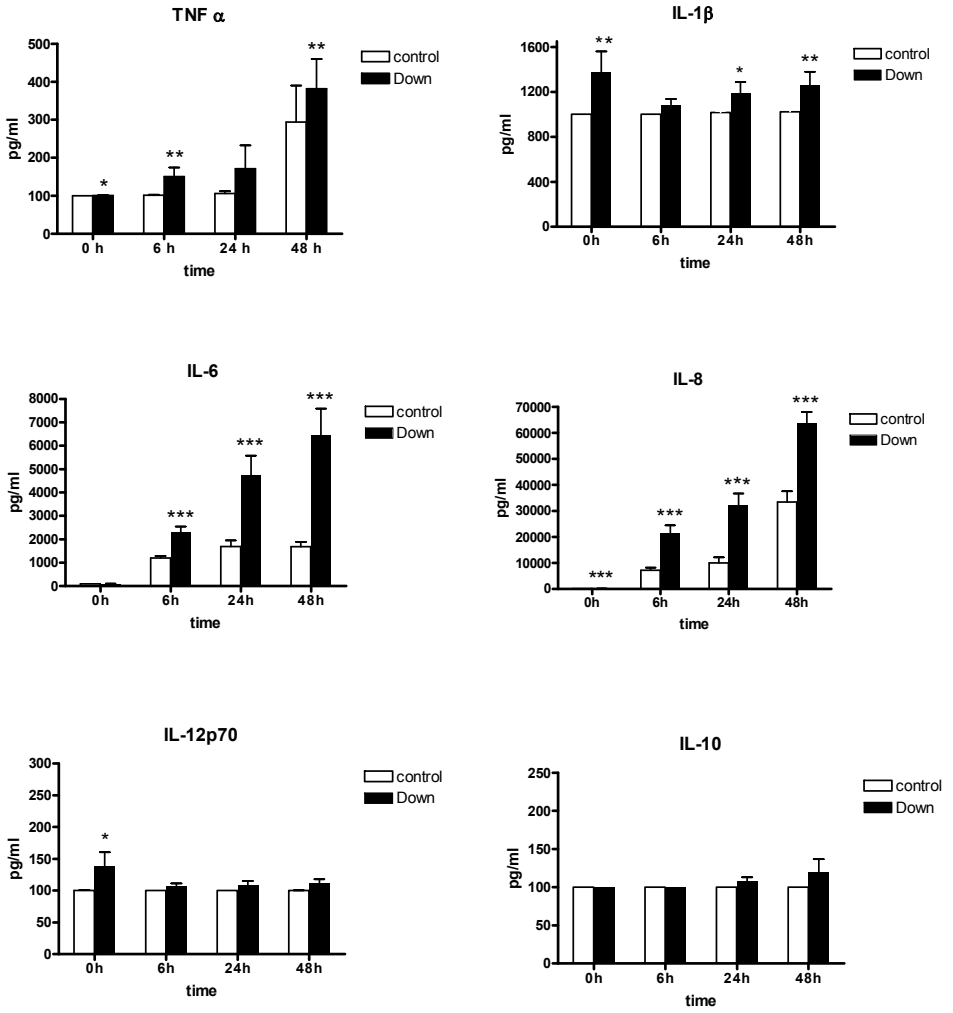

Figure 4.1 Cytokine levels, mean ± SEM, of Down syndrome children (closed bars) and controls (open bars) after stimulation with live influenza A virus.
*p<0.05, **p<0.01, ***p<0.001.

presented. TNF-α, IL-1β, IL-6, and IL-8 were significantly higher in DS children at most time points. At these time points no significant differences were found between the two groups in the levels of IL-10 and IL-12p70. IFN-α and IFN-γ levels at 6, 24 and 48 hours were significantly higher in DS children at most time points, as is shown in Figure 4.2. P-values are summarized in Table 4.1.

Viral RNA copies

No differences were found between the DS group and the sibling group in viral RNA copies at t= 6, 24 and 48 hours after infection with influenza A (Figure 4.3).

Table 4.1 Cytokine levels (pg/ml), mean ± SEM, of Down syndrome children and controls after stimulation with live influenza A virus

		Down syndrome (mean ± SEM) pg/ml	Controls (mean ± SEM) pg/ml	p-value
TNF α	t=0	101.05 ± 0.51	100.00 ± 0.00	0.029 *
	t=6 h	150.26 ± 24.09	101.46 ± 1.46	0.001 **
	t=24 h	171.41 ± 61.03	105.93 ± 5.93	0.060
	t=48 h	381.90 ± 78.22	293.87 ± 96.19	0.003 **
IL-1β	t=0	1374.90 ± 185.35	1000.00 ± 0.00	0.005 **
	t=6 h	1085.16 ± 51.15	1000.00 ± 0.00	0.091
	t=24 h	1193.05 ± 94.33	1016.43 ± 16.43	0.033 *
	t=48 h	1261.63 ± 117.52	1022.02 ± 22.02	0.002 **
IL-6	t=0	103.62 ± 3.38	100.00 ± 0.00	0.174
	t=6 h	2274.03 ± 268.44	1205.39 ± 81.38	0.000 ***
	t=24 h	4722.22 ± 851.63	1689.27 ± 266.02	0.000 ***
	t=48 h	6445.77 ± 1144.70	1684.80 ± 204.30	0.000 ***
IL-8	t=0	165.23 ± 39.74	100.00 ± 0.00	0.000 ***
	t=6 h	21494.90 ± 2916.49	7194.67 ± 1058.94	0.000 ***
	t=24 h	32006.07 ± 4639.25	10015.63 ± 2157.80	0.000 ***
	t=48 h	63517.95 ± 4553.98	33414.49 ± 4210.21	0.000 ***
IL-12p70	t=0	139.48 ± 20.96	100.39 ± 0.39	0.021 *
	t=6 h	107.11 ± 4.28	100.00 ± 0.00	0.091
	t=24 h	108.90 ± 6.32	100.00 ± 0.00	0.086
	t=48 h	110.87 ± 7.24	100.33 ± 0.33	0.194
IL-10	t=0	100.00 ± 0.00	100.00 ± 0.00	1.000
	t=6 h	100.00 ± 0.00	100.00 ± 0.00	1.000
	t=24 h	107.71 ± 5.37	100.00 ± 0.00	0.086
	t=48 h	120.43 ± 16.27	100.00 ± 0,00	0.094
INF-α	t=0	649.53 ± 201.93	158.17 ± 43.41	0.151
	t=6 h	596.72 ± 209.49	137.24 ± 29.70	0.017 *
	t=24 h	1559.35 ± 373.06	274.99 ± 56.25	0.002 **
	t=48 h	1880.67 ± 393.32	321.47 ± 68.62	0.000 ***
INF-γ	t=0	1804.37 ± 585.94	342.57 ± 139.08	0.051
	t=6 h	1983.36 ± 785.18	326.09 ± 128.87	0.175
	t=24 h	1744.67 ± 660.07	254.11 ± 86.74	0.062
	t=48 h	1887.51 ± 740.64	275.56 ± 92.57	0.023 *

*p<0.05, **p<0.01, ***p<0.001.

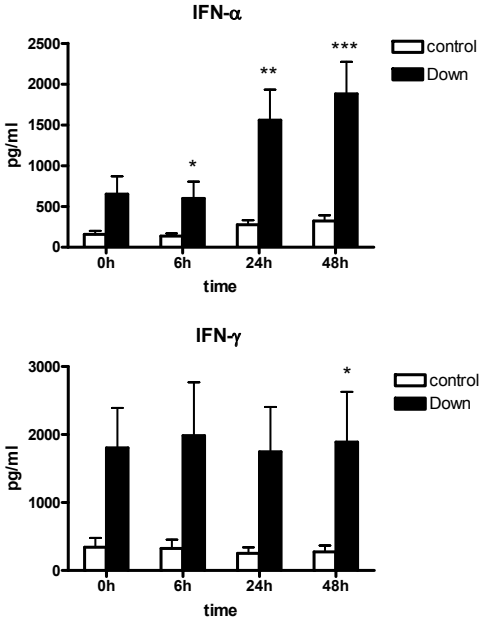

Figure 4.2 Interferon levels, mean ± SEM, of Down syndrome children (closed bars) and controls (open bars) after stimulation with live influenza A virus.
*p<0.05, **p<0.01, ***p<0.001.

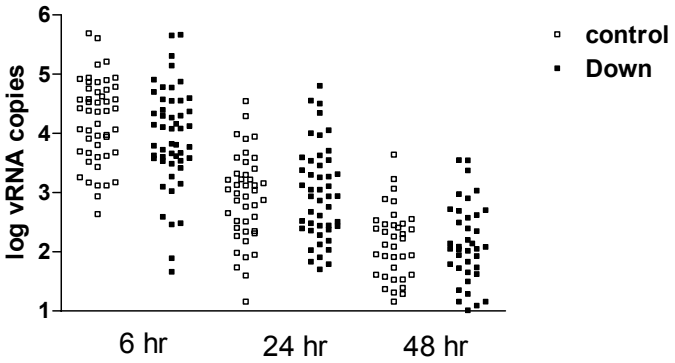

Figure 4.3 Log vRNA copies of DS children (solid squares) and controls (open squares) after stimulation with live influenza A virus at several time points.

Discussion

In this study we demonstrate for the first time in DS children upon ex-vivo stimulation of whole blood with live influenza A virus that TNF-α, IL-1β, IL-6, IL-8 and IFN-α concentrations were significantly higher in comparison to their healthy siblings. IFN-γ

levels were already higher at baseline and did not increase upon stimulation with influenza virus.

Influenza A virus, a single-stranded RNA virus, is a human respiratory viral pathogen which can interact with Toll-like receptors (TLRs) such as TLR3 and TLR7 present on innate immune cells such as monocytes/macrophages, dendritic cells or respiratory epithelial cells. After binding to these receptors this interaction causes an antiviral immune response, stimulating the cells to produce pro-inflammatory and chemotactic mediators, and type I interferons (IFN-α and IFN-β).[28-30] Since we have used whole blood stimulation at 6, 24 and 48 hours, the production of the inflammatory mediators will be mainly by innate immune cells such as monocytes, macrophages and dendritic cells. NK and T cells are unlikely to contribute to the inflammatory response, since type II interferon (IFN-γ) does not increase above baseline levels. We didn't stimulate with T cell mitogens because in our previous work evaluation of T cell function via proliferation to mitogens has not demonstrated a difference between DS and controls (data not shown).

The severity of a viral infection depends on microbial factors as well as host factors. In the host, the balance between pro-inflammatory and anti-inflammatory mechanisms is important in the outcome of a viral infection. Predominantly pro-inflammatory mechanisms cause viral clearance but also tissue damage.[31] Predominantly anti-inflammatory mechanisms do not clear the pathogen,[32] with the possibility of a persistent subclinical infection. Ideally, both mechanisms are balanced: hence the virus is cleared, with protection to re-infection and minimal tissue damage in the host.[33]

In our study the DS children indeed showed an enhanced pro-inflammatory cytokine response and no difference in IL-10 levels between the two groups. These findings point towards a dysbalance between pro-inflammatory and anti-inflammatory mechanisms in DS. This pro-inflammatory cytokine storm may have beneficial effects in an attempt to clear influenza A virus. However, our data indicate that the decrease of viral RNA copies in time was equal in both children with DS and healthy siblings, indicating that viral clearance is similar for both groups. Although replication within the respiratory tract might be different for children with DS, we can rule out the possibility that the increased production of pro-inflammatory cytokines is due to variations in viral RNA copies in our cultures at any time point. Together, these observations point towards an intrinsic difference between leukocytes from children with DS and healthy siblings rather than a difference in viral load. These high cytokine levels may, on the other hand, lead to excessive inflammation, extensive tissue

damage and a more severe clinical course of the viral infection in DS.[17,33] This is also in accordance with earlier reports of an early hyperactivation state of inflammatory response by TNF-α and type I interferon in human viral pneumonia caused by avian influenza virus H5N1, compared to the seasonal influenza A virus H1N1.[34,35]

We also investigated the interferon response to influenza A virus in DS, because both type I and type II interferon are key mediators in the host defense against viral infections. In addition, several interferon receptor genes are localized on chromosome 21[36] and also DS monocytes and fibroblasts demonstrate an increased sensitivity to the effect of interferon.[37,38] The levels of IFN-α and IFN-γ were significantly higher in DS, compared to the control group. Surprisingly the IFN-γ levels in DS were already elevated before incubation with influenza A virus, indicating a permanent hyperactivation state of IFN-γ in DS. Our data confirm the findings by Torre et al., who showed that IFN-γ production is higher in adults with DS.[39] Whether these higher IFN-γ levels reflect a dysregulation of IFN-γ production in DS or continuous viral infections in DS is as yet unknown. Despite higher interferon production, the decrease of viral RNA copies in time appeared to be similar in both the DS group as in the control group, so an impaired viral clearance is not likely to be an additional factor in DS to explain the high IFN-γ levels we found.

In this study, we chose healthy siblings, as closely age-matched as possible, as controls for the DS children in order to minimize genetic, environmental and age-related differences. However, there are several limitations of this study: firstly, the peripheral blood levels of the cytokines may not correlate with cytokine levels in the local tissues i.e. in the lung, where the actual infection is taking place. In future studies it is therefore important to evaluate the local cytokine levels in the lung during a viral respiratory tract infection in children with DS, performing a bronchoalveolar lavage. A second limitation of this study is the use of A/PR/8/34, a human influenza A strain that was adapted to grow in mouse lung and therefore less representative of other human influenza strains. Further research is warranted to address the contribution of airway epithelial cells in response to human influenza strains.

Conclusion

Children with DS have an altered innate immune response to a viral stimulus, which includes the production of higher levels of pro-inflammatory cytokines. This may explain the more severe clinical course of viral infections they undergo.

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