Severe RSV infections in children with Down syndrome: the contribution of an impaired immune system

Beatrijs L.P. Bloemers

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SEVERE RSV INFECTIONS IN CHILDREN WITH DOWN SYNDROME:
THE CONTRIBUTION OF AN IMPAIRED IMMUNE SYSTEM

Ernstige RSV infecties bij kinderen met Down syndroom:
de bijdrage van een verstoord immuun systeem
(met een samenvatting in het Nederlands)

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Beatrijs Lydeke Pieternel Bloemers
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Promotoren: Prof. dr. J.L.L. Kimpen
Prof. dr. A.M. van Furth

Co-promotor: Dr. L.J. Bont

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Voor mammie

De mooiste bloem wordt het eerst geplukt

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VERBIER
VERBIER

Chapter 1

General introduction

Verbier (1500m)
– Les Ruinettes (2200m)

DOWN SYNDROME

Down syndrome (DS) is the most common chromosomal abnormality of live born children. Although prenatal screening for DS has improved over the years, this has not resulted in a decrease of the prevalence of DS. [1] In the Netherlands the incidence of DS is 1 per 650 live born children, which means that approximately 300 children with DS are born per year. [2] Down syndrome was first described by John Langdon Down in 1866 who observed the characteristic physical appearances such as broad nasal bridge, downslanted eyes, in combination with hypotonia and mental retardation. In the 1930s it was already speculated that DS could be due to chromosomal abnormalities. But it took until 1959 that a third chromosome 21 (trisomy 21) was found to be the cause of this syndrome. [3-6] Cases of DS due to translocation and mosaicism were described over the next three years. [7;8] In addition to physical appearances and mental retardation, DS is associated with multiple congenital abnormalities and other co-morbidity (Table 1). In newborns with DS the most common abnormalities are gastro-intestinal disease, eg duodenal atresia, congenital diaphragmatic hernia or Hirschsprung disease, and congenital heart disease of which ventricular septal defect and atrio-ventricular septal defect are the most common conditions. [2;9-11] Infants with DS often present with celiac disease [12-14] or hypothyroidism. [15-17] Growing older children with DS have an increased incidence of diabetes mellitus [18;19], a 10-20 fold risk of leukaemia [19;20] and eventually Alzheimer's disease already before the age of sixty. [21;22] At all ages an increased incidence of respiratory morbidity and mortality is found in individuals with DS. [11;23-29]

Table 1. Co-morbidity in individuals with Down syndrome.

Co-morbidity	Incidence in DS
Gastro-intestinal disease [2;9;10]	5-10%
Congenital heart disease [2;9;11]	50-55%
Celiac disease [12-14]	5-20%
Hypothyroidism [15-17]	3-35%
Diabetes mellitus [18;19]	0.5-10%
Leukaemia [19;20]	1.0%
Alzheimer's disease before age 60 [21;22]	30-55%

IMMUNE FUNCTION

Over the years it has been suggested that the increased incidence of leukaemia, celiac disease, hypothyroidism, diabetes mellitus, and Alzheimer's disease might be explained by an impaired immune system in patients with DS. [30-36] The high incidence of respiratory morbidity in children with DS might be (partially) explained by an aberrant immune

system as well. We have focused on the innate immunity and T-cells in children with DS, and therefore an introduction on only these cell types of the immune system is provided.

Innate immunity

The innate immune system is the first to respond during infections, not depending on immune recognition by lymphocytes. Pathogen-associated molecular patterns (PAMPs) are recognized by the innate immune system, which results in a first-line of defense against pathogens. Peripheral blood consists of multiple innate immune cell types, including granulocytes, monocytes, natural killer (NK) cells, invariant natural killer T-cells (iNKT-cells) and dendritic cells (DCs).

Granulocytes, also referred to as polymorphonuclear leukocytes, are characterized by the presence of granules in their cytoplasm. Based on staining characteristics, they can be divided in neutrophils, eosinophils, and basophils. Granulocytes participate in phagocytosis, can cause an inflammatory response by releasing granules that contain histamine and regulate other immune cell functions acting as antigen presenting cells. Monocytes compose approximately 2-8% of white blood cells. CD16 is used to differentiate between two major sub-populations within monocytes: classical or regular monocytes ($CD14^{+}CD16^{-}$) and non-classical or "proinflammatory" monocytes ($CD14^{dim}CD16^{+}$). [37] Although the true function of the two sub-populations is not fully understood, it has been suggested that $CD14^{dim}CD16^{+}$ monocytes have pro-inflammatory properties [38;39], superior antigen presenting cell activity [40] and direct antibacterial activity in the tissue [41]. In addition, $CD14^{+}CD16^{-}$ monocytes are suggested to have a scavenging function in the blood and may contribute to removal of apoptotic neutrophils and debris. NK-cells belong to the lymphocyte population, but express CD16 (FcyRIII) and CD56 instead of T-cell or B-cell receptors and therefore are part of the innate immunity with its aspecific killing. These cells play a major role in viral infections releasing granules with perforin and granzyme that cause apoptosis of the target cell. NK cells depend on activating signals by cytokines or through activation of their Fc receptor or activating and inhibitory receptors. A very small subset, only 0.2% of all T-cells in peripheral blood, is iNKT-cells, characterized by the expression of an invariant TCR (Vα24 and Vβ11). iNKT-cells can produce cytokines within hours, thereby initiating the immune response. iNKT cells can further contribute to the initial anti-viral immune response by inducing activation of NK and T-cells. [42;43] Dendritic cells are professional antigen presenting cells with two major subclasses that arise from both myeloid and lymphoid progenitors within the bone marrow. They migrate via the blood into tissues throughout the body and to peripheral lymphoid organs. Myeloid DCs (mDCs), characterized by the expression of high levels of CD11c on their surface, have a direct role in antigen presentation and activation of naïve T-cells. They induce important signals like up regulation of co-stimulatory molecules and cytokine responses (IL-12) upon activation. During viral infection mDCs promote a Th1-

type response of CD4 T-cells. Plasmacytoid DCs (pDCs), which do not express CD11c, are also professional antigen presenting cells responding to viral infections, but without much effect on naïve T-cells. Upon activation pDCs produce primarily interferon-alpha, which recruits activated macrophages to the site of inflammation.

Adaptive immunity

T-cells

Thymus

Hematopoietic precursors derived from the bone marrow enter the thymus from the blood to complete their development into T-cells. Different processes take place in thymocytes to become mature T-cells. Distinct T-cell receptors (TCR) are developed through gene rearrangement. Positive and subsequently negative selection eliminates T-cells with weak interaction between their TCR and MHC molecules or that are auto-reactive. In addition to TCR development, thymocytes undergo T-cell lineage commitment resulting in mature single positive $CD4^+$ or $CD8^+$ T-cells in the absence of cell division. Once mature, T-cells are no longer susceptible to apoptosis, instead proliferate upon antigen receptor triggering and are released into the peripheral blood. [44]

With ageing thymic involution is seen, affecting both T-cell progenitors and the thymic microenvironment. [45] Thymic output declines significantly with age, but the thymus retains limited activity at least up to the age of 60 years. [46;47] Despite the decline in number and function of T-cells with age, individuals do not become severely immunodeficient, although the elderly are more prone to infections and vaccines are less effective. [48] Children thymectomized at an early age show a significant decrease in T-cell number and function as well, but that does not result in clinical immunodeficiency. [49-51] In contrast, children with DiGeorge syndrome, which have complete absence of a thymus from birth, suffer from severe T-cell immunodeficiency. [52] These data suggest the capacity of secondary peripheral generation of T-cells.

Naïve T-cells

Naïve T-cells are antigen-inexperienced. They are defined by their expression of CD45RA, CD62L, CD27, CD28, and CCR7. [53] Naïve T-cells can produce IL2, but not interferon-γ (IFNγ) and IL-4, which are a hallmark of antigen-experienced memory/effector T-cells. Their TCR repertoire is highly diverse since they have not undergone clonal expansion. At least three distinct subsets of naïve T-cells exist: naïve T-cells that have been recently produced and emigrated from the thymus (recent thymic emigrants, RTE) and two naïve T-cell subsets that have been residing in the naïve pool for longer time. Based on phenotypic markers differences in these naive $CD4^+$ T-cells subsets can be distinguished.

[54-56] Naive CD31 positive and PTK7 positive $CD4^+$ T-cells are considered to be the subset most proximal to the thymus and MHC /selfpeptide ligand induced proliferation of these cells has been suggested to lead to loss of PTK7 and subsequently CD31 expression. IL-7 preferentially induces proliferation of the CD31 positive subset thereby down modulating CD127 expression but not inducing any other phenotypical changes. [57] In contrast, TCR engagement may lead to differentiation into loss of CD31 expression. [57;58] Although all RTEs should express CD31, not all cells expressing CD31 represent RTEs. Absolute numbers and frequency of $CD31^+$ naïve T-cells within the naïve T-cell pool decrease dramatically with age, while absolute numbers of $CD31^-$ naïve $CD4^+$ T-cells remain stable in peripheral blood over time. [58] In conclusion the naïve T-cell pool exists of three subsets: RTE ($PTK7^+$ $CD31^+$) and two residual naïve subsets that are probably the result of respectively IL-7 induced proliferation ($PTK7^-$ $CD31^+$) and TCR-driven peripheral expansion ($PTK7^-$ $CD31^-$).

Homeostasis of the naïve T-cell pool

Throughout life relatively constant numbers of naïve $CD4^+$ T-cells are found in the peripheral blood, suggesting a certain homeostasis of the human naive T-cell pool. [59] Thymic output of RTE, peripheral expansion and longevity of existing naïve T-cells have a positive effect on the number of naive T-cells in the peripheral blood. [60] Apoptosis and clonal expansion to a memory or effector phenotype upon antigen stimulation decrease the number of cells in the naive T-cell pool.

RESPIRATORY SYNCYTIAL VIRUS AND RECURRENT WHEEZE

Respiratory syncytial virus (RSV) is the single-most important pathogen in lower respiratory tract infections in infancy. [61] RSV is a single-stranded RNA pneumovirus of 120-300 nm belonging to the family of Paramyxoviridae. In addition, RSV is closely related to (para)influenzaviruses.

Epidemiology

In the first year of life nearly 70% of children are infected by RSV. [62] By the age of 2 years virtually all children have been infected at least once. [61] Most children represent with a runny nose or coughing, but 1-2% of all children become hospitalized because of feeding problems or dyspnoea due to lower respiratory tract involvement. [63;64] Approximately 10% of children hospitalized because of RSV bronchiolitis are severely ill requiring mechanical ventilation in the intensive care unit. [65] Premature born children, children with pre-existing lung disease, e.g. bronchopulmonary dysplasia or cystic fibrosis, children with hemodynamically significant congenital heart disease, and im-

munodeficient children have a higher risk to develop severe lower respiratory tract infections requiring hospitalization. [66] In addition, male gender and certain environmental factors such as smoking and attending day care add to the risk to become hospitalized. In healthy children the peak incidence is in children of 2-3 months of age at the beginning of the RSV season, eg the winter season in moderate climates and the wet season in tropical regions. [67;68]

Pathophysiology

It is currently unclear why a mild course of disease upon RSV infection is seen in most children, while in a small group it can become quite severe. Over the years it has been widely discussed whether (more severe) disease is caused by the virus due to direct cytopathology or by the host itself reacting with an overwhelming immune response. In a healthy subject infection with RSV leads to an effective combined innate and adaptive immune response resulting in clearance of the virus before it has caused substantial cytopathology and only mild collateral damage due to the host inflammatory response. In an immunocompromised setting RSV causes high morbidity and mortality due to direct cytopathology and prolonged shedding of the virus has been shown. [69-73] In this setting the virus itself seems to be the main cause of severe disease. On the other hand, vaccine studies in the 1960s showed that immune mediated pathology without pronounced virus induced pathology could result in severe RSV disease as well. [74-76] Although the exact mechanism of severe RSV disease is unclear, several hypotheses have been proposed. Genetic factors [77-82], environmental factors [83-85], virus strain [86-88] and load [89-92] might play a role in disease severity, but conflicting data have been reported. The young age at which most severe disease is seen, suggests a role for maturation of the immune response. An immature immune system may result in ineffective viral clearance, or alternatively in an undesired overwhelming or biased response. A bias towards Th2 type of responses has been shown in the immature immune system of children [93-96] and this same type of response was shown in animal models of enhanced disease upon primary RSV infection after vaccination.[97] RSV challenge after vaccination with formalin inactivated RSV in mice was shown to involve a large eosinophilic influx, a typical Th-2 type of response. A large eosinophilic influx was also shown after primary RSV infection in mice. [98] However, during the acute phase of RSV infection mice showed a pre-dominant Th-1 cytokine response. Whether these murine models reflect the human response to RSV infection is a matter of debate, since some studies report increased levels of Th-2 cytokines [99], while others did not find skewing of the Th-1/Th-2 balance. [100] Many studies have focused on the adaptive immunity, but more recent studies suggest an important role for the innate immunity as well. [81] Associations with genetic differences in cytokine and cytokine receptors and severe RSV infections have been found. Single-nucleotide polymorphisms in the innate immune genes *VDR, JUN, IFNA5* and *NOS2* are strongly associated with RSV

bronchiolitis. [101] Tulic et al have shown that both in a transfection model of epithelial cells and in an in vitro model of PBMCs, a functional TLR 4 polymorphism leads to an aberrant signal transduction and gene transcription in response to RSV and lipopolysacharide. [102] In conclusion, the pathophysiology of severe RSV infections is not fully understood, but it is clear that both innate and adaptive host immune responses determine the outcome of infection.

Treatment and prevention

No treatment options are available for RSV infection except for supportive care, eg naso-gastric feeding, oxygen supply or mechanical ventilation in case of severe RSV-associated disease. Since the failure of the first vaccination trial in the 1960s many attempts have been made to develop a vaccine for RSV, but until now none has been shown to be safe and effective. [74-76] At the same time, passive immunization has been developed, first intravenous immunoglobulins [103] and subsequently intramuscular injections with monoclonal immunoglobulins (palivizumab), which have been proven to be safe and effective in preterm children [104] and children with CHD. [105] Due to high costs of these immunoglobulins passive immunization is only approved for administration in the previously described risk groups for severe RSV.

Recurrent wheeze

Wheezing disorders are a common problem in childhood, affecting 10-20% of children in the first three years of life. [106] Wheezing during early life is a heterogeneous disorder and different wheezing phenotypes can be distinguished upon timing of onset (early or late wheeze), persistence (transient or persistent wheeze) and underlying mechanism (viral or allergen induced wheeze). In most children wheezing is transient, spontaneously resolving before school age, while the other half develops persistent wheeze. [107;108]

Viral lower respiratory tract infections are associated with signs of airflow limitation during the acute infection, but also with the subsequent development of long-term wheeze. About half of children hospitalized for severe RSV infection will experience recurrent wheeze in the first two years following hospitalization. [108-111] Similarly, rhinovirus and enterovirus are other viruses frequently associated with subsequent wheeze. [112-114] Controversies in literature exist with respect to the outcome of recurrent wheeze following RSV, referred to as post-bronchiolitis wheeze. Some studies suggest post-bronchiolitis wheeze to be mainly a non-allergic condition with a good long-term prognosis [108;115-117] and studies that suggest that this recurrent wheeze is an early onset of allergic asthma. [111;118-120] It was shown by genetic polymorphism studies that early wheeze (during the first 15 months following RSV bronchiolitis) should be distinguished from late wheeze (at the age of 6 years) following RSV, with early wheeze being distinct from allergic asthma. [101]

Infection of the lower respiratory tract with RSV causes inflammation of the airways, which eventually might result in early wheeze. [121] It was therefore suggested that early anti-inflammatory therapy during the acute phase of RSV infections might prevent early wheeze. Although a moderate and transient beneficial effect on early wheeze following severe RSV infections in non-ventilated children has been shown, overall early-initiated inhaled corticosteroids does not prevent early wheeze following RSV in all hospitalized patients. [122]

OUTLINE OF THE THESIS

This thesis started with the observation by experienced clinicians that severe RSV bronchiolitis, often with a complicated course of disease, was observed more frequently in children with DS than in other children. However, this observation had not yet been documented in literature and the underlying mechanism was obscure. In the last thirty to forty years several studies have focused on the immune system of patients with DS in an attempt to clarify the clinical problems frequently seen in this specific population. Although multiple congenital abnormalities are associated with DS, respiratory tract infections are an important cause of morbidity frequently seen in these children in daily clinical practice. We have attempted to increase our understanding of the basic functions of the immune defence systems in children with DS and to translate it to their role in virus-associated respiratory morbidity.

Aim

To study whether children with Down syndrome have an increased risk of severe RSV infections and how this could be mediated by impaired innate and adaptive immune system.

Hypotheses

1. The incidence of severe RSV bronchiolitis requiring hospitalization in children with DS without co-morbidity is increased
2. A history of RSV bronchiolitis in children with DS does not add to the risk of recurrent wheeze
3. Children with DS have abnormal number of innate immune cells in the peripheral blood
4. Low naïve T-cell numbers in children with DS is fully explained by insufficient thymic output

First, the literature on respiratory tract infections and immunology in patients with DS was reviewed **(Chapter 2)**. In **Chapter 3**, the frequency of cells of the innate immune system in the peripheral blood was studied with a specific focus on $CD14^{dim}CD16^{+}$ monocytes. In order to improve our understanding of low naïve T-cells in children with DS we studied T-cell dynamics (**Chapter 4**). The relative contribution of thymic insufficiency, peripheral generation and loss to decreased numbers of naïve T-cells in children with DS was compared to age-matched controls.

Although respiratory morbidity is a common problem of children with DS addressed in daily clinical practice, only a few reports have described the extent of this issue. In addition to the low quantity of this type of studies, most of them only report retrospective results. Therefore, a prospective national birth cohort of children with DS from the Netherlands was studied to estimate the true incidence of RSV-associated hospitalization **(chapter 5)**. The relevance of this study is that RSV prevention might be possible using immunoprophylaxis, as shown previously in preterm children and children with congenital heart disease.

In order to determine whether children with DS have pre-existent susceptibility to severe viral respiratory morbidity underlying the high incidence of RSV-associated hospitalization, an epidemiological approach was taken to compare the development of recurrent wheeze in children with DS with and without a history of RSV bronchiolitis **(chapter 6)**.

Chapter 7 gives an outline of our main findings which are discussed in detail and translated to use in clinical practice. A summary of this thesis in English and Dutch is provided.

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Chapter 2

Increased risk of respiratory tract infections in children with Down syndrome: the consequence of an altered immune system

Beatrijs L.P. Bloemers
Chantal J.M. Broers
Louis Bont
Michel E. Weijerman
Reinoud J.B.J. Gemke
A. Marceline van Furth

Microbes and Infection, in press

Tortin (2050m)
– Col des Gentianes (2950m)

ABSTRACT

Down syndrome (DS) is the most common chromosomal abnormality among live-born infants. Respiratory tract infections are the most important cause of mortality in individuals with DS at all ages. In recent decades several studies have been performed to elucidate abnormalities of the immune system in DS. However, the influence of the immune system on the occurrence of respiratory tract infections in these children has never been reviewed.

INTRODUCTION

Down syndrome (DS) is the most common chromosomal abnormality among live-born infants. In Europe DS accounts for 8% of all registered cases of congenital anomalies. In a recent study conducted by our Down Syndrome Study Group the prevalence of DS in the Netherlands was determined to be 16 per 10,000 live births and thus is much higher than suggested in previous literature. [1] DS is characterized by a variety of dysmorphic features and congenital malformations, including congenital heart disease (CHD) and gastrointestinal disease. In addition, DS is associated with various immunological impairments. Leukemia and auto-immune diseases like hypothyroidism, celiac disease and diabetes mellitus are more prevalent in these children. [2] Respiratory tract infections (RTIs) in children with DS are a common problem encountered in daily clinical practice but evidence in literature is sparse. [3-6]

Life expectancy in children with DS has increased significantly in the last decade. However, neonatal and infant mortality in DS in the Netherlands is still 5 and 8 times higher than in children without DS, respectively (1.65% vs 0.36% and 4% vs 0.48%). [1] Important causes for increased mortality in DS are congenital heart disease, other congenital anomalies (e.g. of nervous system, respiratory system, gastro-intestinal tract, genito-urinary system and musculoskeletal system), leukemia, testicular cancer and sepsis. In addition RTIs are still the most important cause of mortality in DS at all ages. [7-11]

Over the years, it has been suggested that the increased incidence of leukaemia, celiac disease, hypothyroidism, and diabetes mellitus might be explained by an impaired immune system in patients with DS. A high incidence of respiratory morbidity in children with DS might be (partially) explained by an aberrant immune system as well. We have reviewed the literature on RTIs to support the clinical finding of a high incidence of respiratory morbidity in children with DS. Next, we have reviewed the immunologic literature on children with DS to clarify the role of the immune system in the respiratory morbidity in this specific population.

RESPIRATORY TRACT INFECTIONS

Children with DS have an increased risk of RTIs. RTIs can be divided in infections of the upper respiratory tract (URTI) (e.g. sinusitis, middle ear infections, rhinitis, tonsillitis, pharyngitis, laryngitis subglottica) and lower respiratory tract (LRTI) (e.g. pneumonia, bronchiolitis), which can be of diverse pathogenic origin (e.g. viral, bacterial, fungal or a combination of these).

Although data on the frequency of URTI in children are not exactly known and may vary in different studies because of different definitions and criteria by which they

are assessed, the frequency of URTIs in children with DS seems increased compared to healthy controls: 12% have more than 3 URTIs in 12 months. [5] The most frequently described infections include pharyngitis in 27% and otitis media with effusion in 55%. [5;6] Abnormal anatomy of the upper respiratory tract may predispose children with DS to (chronic) URTIs. Stenotic ear canals, present in 40-50% of the newborns with DS, results in cerumen impaction. [12] Midface hypoplasia is common in these children as well, with smaller and abnormally inserted Eustachian tubes, and smaller nasal area as well as nasal sinuses. This, in combination with dysfunction of Eustachian tubes, may lead to accumulation of middle ear fluid and obstruction of airflow, making these children prone to otitis media. Hypoplasia of the nose and sinuses contributes to nasal obstruction, rhinorrhea and sinusitis. Hyperproduction of mucus was shown for most children with DS in a study performed by Piatti et al., but the ultrastructure and functions of the nasal cilia were normal. [13]

Lower respiratory tract pathology is the main cause of hospitalization and the most frequent cause for admission to the pediatric intensive care unit in children with DS. [4] Some children with DS and LRTI require intubation and mechanical ventilation. Children with DS were reported to have a higher incidence of acute lung injury and acute respiratory distress syndrome when they are mechanically ventilated in acute LRTI. [14] Acute lung injury is known to be associated with elevated rates of apoptosis of leukocytes and epithelial cells. [15] In DS an increase in the apoptosis of granulocytes has been observed [16] which might be a factor in DS contributing to a higher rate of acute lung injury.

A few case reports have been published on LRTIs in children with DS that were caused by uncommon microorganisms or that showed an uncommon course of disease: Cant et al. described four cases of bacterial tracheitis in children with DS, of which three were caused by *Haemophilus Influenzae.* [17] These children were severely ill and had to be intubated and mechanically ventilated. One report described a child with DS that died because of pneumonia caused by *Bordetella bronchiseptica*, which normally causes RTIs only in animals. [18] Finally, Orlicek has reported on three children with DS under the age of 5 with a severe course of pneumonia caused by *Mycoplasma pneumoniae*, a microorganism that in the general population uncommonly produces such a serious infection. [19]

Besides these few case reports on rare pathogens or uncommon course of disease, there have been hardly any studies on the association of more common respiratory pathogens and severe LRTI in children with DS. *Respiratory syncytial virus* (RSV) is the most important cause of severe LRTI in infants and young children worldwide, leading to hospitalization in many cases. DS is an independent risk factor for severe RSV-LRTI, resulting in a 10-fold increase in the risk of hospitalization for RSV LRTI. [3] CHD, present in 40-60% of children with DS, is associated with an increased risk of hospitalization for RTIs, of which RSV is the most common pathogenic cause. [20] Children with DS with hemodynamically significant CHD have a more than twofold higher risk of hospital

admission because of RTIs compared to controls with hemodynamically significant CHD without DS. [21]

In addition to the upper respiratory tract, anatomical abnormalities of the lower respiratory tract, such as laryngo- and tracheomalacia have been shown as well in children with DS. [22] Two groups have reported disturbed lung growth in children with DS that results in alveolar and pulmonary hypoplasia. [23-25] These abnormalities might lead to a different airway physiology with increased susceptibility to RTIs in children with DS compared to controls.

Children with DS are known to suffer from generalized hypotonia that may result in swallowing dysfunction and subsequently silent (micro) aspiration. [26] Recurrent aspiration of thin fluids is associated with an increased incidence of LRTIs. However, a study in children with neurologic impairment performed by Weir et al. showed that the diagnosis of DS was significantly associated with pneumonia, but swallowing dysfunction in these children did not have an additive effect on the risk of pneumonia. [27]

In conclusion, children with DS have an increased incidence of RTIs which might be associated with congenital heart disease, abnormal airway anatomy and physiology, hypotonia, and aspiration.

IMMUNOLOGY

In the last thirty to forty years several studies have focused on the immune system of patients with DS in an attempt to clarify the clinical problems frequently seen in this specific population. Although multiple congenital abnormalities are associated with DS, RTIs are an important cause of morbidity frequently seen in these children in daily clinical practice. In the following paragraphs, we have attempted to increase our understanding of the functions of the immune defence systems in children with DS and to translate it to their role in respiratory morbidity.

Innate immunity

Cell numbers

The innate immunity is very important in the first-line defense against micro-organisms. Although children with DS have a high incidence of RTIs, innate immune responses have only been partially studied. Over the years different cell surface molecules have been used to describe different innate immune cells, which make it difficult to compare more recent with previous studies. The number of $CD16^{+}CD56^{+}$ natural killer (NK) cells is decreased in children with DS. [28;29] By contrast, in adults with DS this subset was shown to be significantly increased. [28] The exact function of CD57 on NK cells is not

fully clear, but $CD16^{-}CD57^{+}$ cells were suggested to have low NK activity compared to $CD16^{+}CD56^{+}$ cells. In both children and adults with DS $CD16^{-}CD57^{+}$ cells are significantly increased. [28] Although functional studies have been performed with other innate cells such as polymorphonuclear granulocytes and monocytes, none of them have focused on absolute numbers. Neutrophils are reported to normally express surface markers (eg CD11a, CD11b, CD16 and CD18) in DS. [30] Invariant natural killer T-cells (iNKT-cells) have never been studied in DS up to date.

In conclusion, accurate information on numbers of most innate immune cells is not available, except for $CD16^{+}CD56^{+}$ NK cells which are decreased in individuals with DS compared to controls.

Cell function

Chemotactic migration of polymorphonuclear leukocytes (PMN) and mononuclear phagocytes is found reduced in DS. [16;30-32] This finding was suggested to be secondary to either an intrinsic defect of the leukocytes of DS (due to a shorter half-life), or enzymatic defects, or shifts in the migrating subpopulations of leukocytes. In contrast, random mobility, without chemotactic gradient, is normal in DS for leukocytes and mononuclear phagocytes. [30-32]

Some authors describe that PMN phagocytosis in children with DS is comparable to controls. [30;33] Others, like Rosner et al. have shown decreased in vitro phagocytic ability of peripheral blood neutrophils to ingest live *Candida albicans* and decreased neutrophil adhesiveness in DS compared to controls. [34] No differences in the oxidative burst of PMN leukocytes have been established in children with DS compared to controls. [30] Peroxidase and periodic-acid/Schiff activity in leukocytes is normal as well. [34] Although, a small decrease in superoxide production by isolated neutrophils has been described. [16] Adults with DS have increased percentages of apoptotic neutrophils and eosinophils, both spontaneously and anti-fas antibody induced. [16] GM-CSF and IL-5, cytokines that are reported to support the survival and activation of granulocytes, have less protective effect on apoptosis in DS than in controls. Fas and bcl-2 expression did not show any differences between DS and controls.

Studies on NK activity in DS have also shown contrasting results. Nurmi et al. showed slightly higher NK activity in adult patients with DS, both in peripheral blood mononuclear cells (PBMCs) and monocyte depleted PBMCs. [35] Lower NK cytotoxic activity compared to controls has been shown in children and adults with DS by others. [28;36;37] Nair et al. have shown that during NK- cytotoxicity assays in children with DS lower levels of IFN are produced by lymphocytes against target cells in vitro. [37] This NK activity could be up regulated in DS by adding IL-2 or PHA to the culture, but not up to levels of healthy controls. However, in adults with DS NK activity could reach similar levels compared to controls by using peripheral blood lymphocytes (PBLs) preincubated with IL-2, IFN-β or

IFN-γ. [28] In DS subjects, no correlation between numbers of NK cells and NK activity has been observed.

IFNAR1 and 2, the genes encoding for the interferon α/β receptor, which binds type I interferons, are located on chromosome 21. [38;39] Because of an increased expression of the IFN receptor, Trisomy 21 patients may have enhanced sensitivity to the antiviral effects of interferon. Trisomic fibroblasts were shown to have a three times higher response to both virus-induced and PHA-induced human interferon. [38] Together with an antiviral effect, interferon has several quite diverse effects as well. Epstein et al. have shown an increased sensitivity of DS monocytes to the inhibiting action of interferon on lysosomal enzyme activity, a measurement of monocyte maturation. [40] Although the number of patients in the study was small, this effect was hypothesized to override the antiviral effect and therefore to result in a reduced rather than an increased ability to response to infectious agents in DS.

In conclusion these studies show conflicting results on a more or less disturbed PMN function resulting in decreased chemotaxis, normal or decreased phagocytosis and increased apoptosis. Overall most studies in DS suggest a decreased NK cell activity in vitro, although this depends on the presence of cytokines and seems to improve with age. The results might be conflicting due to the age of patients studied, techniques used to separate cell populations and variations in DS individuals as well.

Adaptive immunity

T-cells

Thymus

Several groups have proposed that T-cell abnormalities found in children with DS might be explained by an abnormal thymic function and suggested that this dysfunction was the consequence of early senescence of the immune system. Over the years, several studies have been performed focusing on thymic deficiency in DS. Morphological and immunohistochemical studies of DS thymus have shown comparable histologic alterations. [41-45] Children with DS, differing in age from 1 day old up to 4 years of age, showed moderate to severe cortical thymocyte depletion with decreased thickness of the cortex compared to children without DS and from a poor demarcation to a complete disappearance of the corticomedullary junction. In addition, all studies showed enlarged Hassal's corpuscles in DS with cystic changes in the majority of cases and fibrosis in 46 to 77%. Levin et al. concluded that age was not a determining factor, since newborns already showed marked alterations. [41] In contrast, Larocca et al. defined three different groups according to severity of the thymic lesions and found that this was roughly related to age. [45] No definite conclusions can be drawn since groups were small and age

distribution was not equal. No extrinsic factors (eg stress) have been found to explain differences between children with and without DS when thymus pathology was combined with clinical data. [41;42] In conclusion, these histopathologic results are compatible with accelerated involution and atrophy of the thymus as seen in elderly.

Thymic hormones have been studied to provide additional evidence of thymic impairment in DS. Activity of serum thymic factor (FTS), which is suggested to be essential for further differentiation into fully immunocompetent T lymphocytes, has been found to be lower in DS [46;47] Lack of this factor was proposed as the primary cellular immune defect in DS. Fabris et al. showed lower FTS activity as well, with additional higher FTS inhibitory activity in DS compared to controls. [48] However, they also showed that this loss of activity could be restored by zinc-suppletion, and therefore do not support the hypothesis of primary thymic dysfunction.

Studies of thymocytes and subpopulations also have shown marked alterations in children with DS. Proportions of $CD1^+$, $CD3^+$, $CD4^+$ and $CD8^+$ thymocytes are decreased in children with DS. [43;44] Double positive $CD1^+CD3^+$ thymocytes are significantly lower compared to controls. In children with DS, a different distribution of cells with high expression of CD3 (CD3 bright) and low expression (CD3 dim) has been found. Compared to controls, these children have lower percentages of CD3 bright cells (18% versus 43%) and higher percentages of CD3 dim cells (58% versus 36%). [49] In addition, normal proportions of total CD3 expressing cells are found. Further maturation of thymocytes yields surprising differences. [50] Children with DS have higher percentages of CD8 bright thymocytes with lower percentages of CD8 dim cells, compared to controls. However, DS CD4 bright and dim cells are equal to controls. Total percentages of double negative and single positive CD4 and CD8 thymocytes are slightly decreased while the double positive thymocytes are slightly increased. However, differences have not been shown to be significant, probably due to the size of the groups (n=8). These studies suggest that in DS the process of T-cell commitment to either CD4 or CD8 single positive T-cells is present, although perhaps somewhat incomplete. Whether these different levels of expression will result in functional changes in DS has not been studied.

DS thymocytes are able to express TCRαβ. However, a significantly lower proportion of TCRαβ bright cells and a higher proportion of TCRαβ dim cells have been described in these children. The total percentage of thymocytes expressing TCRαβ is slightly lower in children with DS as well. [43;44;49;50] In contrast, normal proportions of TCRγδ expressing thymocytes in DS are described. [44] The findings of a higher percentage of mature single positive thymocytes expressing lower levels of TCRαβ and CD3 suggest a dysfunctional maturation in DS. These studies are partially indicative of a delayed maturation of T cells within the thymus of DS.

Children with DS show slightly lower proliferative responses of thymocytes to IL-4 in vitro. [51] However, TCRγδ thymocytes, the thymocytes most responsive to IL-4, are normal in DS.

IFNγ and TNF have important inhibitory effects of IL-4 induced thymocyte proliferation. [52] In children with DS both IFNγ and TNFα expression are increased, with higher sensitivity to inhibition of the IL4-induced response in vitro. Consequently, T-cell differentiation and maturation might be impaired in DS.

Although the previous studies suggest dysfunctional maturation of thymocytes, this is not reflected directly in PBL. The expression of both CD3 and TCRαβ on PBL in DS is comparable to children with DS. However, both the percentage and absolute numbers of TCRαβ expressing cells is decreased in DS. In contrast, the proportion and absolute numbers of TCRγδ is increased. [53]

In recent years attempts have been made to quantify thymic output by using T-cell receptor excision circle (TREC) content of cells. Lower percentages of TREC positive lymphocytes have been shown in children with DS, which showed an age-related decrease in contrast to healthy controls. [54;55] Low TREC content, however, might rather be explained by lower percentage of naïve T-cells in DS, the main cell type containing TRECs. Higher plasma levels of IL-7, a primary cytokine in T-cell survival and maturation, and IL-15, a primary cytokine in the regulation of CD8 T-cells, are found in children with DS, with normal expression of IL-7Rα. Although DS T-cells show normal responses upon IL7 stimulation, the increased IL7 plasma levels do not result in higher proliferation rates of DS T-cells.

In conclusion, studies on DS thymus have provided evidence of accelerated involution of the thymus, an altered pattern of maturation of thymocytes and indications of inefficient thymic output.

T-cell numbers

Absolute total leukocytes and lymphocytes are significantly lower in children with DS at all ages. [29;56-58] As a consequence absolute numbers of T-cells are lower as well, especially in the first two years of life. With increasing age differences become smaller. As expected, absolute counts of $CD4^+$ and $CD8^+$ T-cell subsets are lower as well. Looking at distribution of lymphocytes, normal percentages of $CD3^+$ T-cells are reported. [56-58] In contrast, increased percentages of $CD8^+$ and decreased $CD4^+$ subsets are described [57], although, according to Cocchi et al only beyond the age of 3 years. [56] The $CD4^+/CD8^+$ ratio is stable with age, but lower in DS than in healthy controls. Further differentiation of $CD4^+$ and $CD8^+$ subsets reveals lower percentages and absolute counts of naïve T-cells in both subsets and increased percentages of memory subsets.[53;55] One study has reported increased percentages of activated (Ia^+) T-cells in DS. [59] This finding might be

explained by the fact that all these patients had known auto-immune diseases such as hypothyroidism and diabetes mellitus.

It can be concluded that children with DS have decreased absolute numbers of actually all $CD4^+$ and $CD8^+$ T-cell subsets, especially in the first two years of life, with a relative increase of $CD8^+$ T-cells and memory subsets.

T-cell function

As described above, most of the $CD3^+$ T-cells in the peripheral blood of DS have a mature phenotype. These cells would be expected to proliferate normally after anti-CD3 stimulation. In contrast, proliferation upon stimulation with anti-CD3 is depressed in children with DS. [60;61] Scotese et al. have shown that children with DS have an aberrant pattern of the signaling pathway after CD3 cross-linking, characterized by the absence of tyrosine phosphorylation of part of the proteins involved in the cascade. In contrast, the gamma chain of the IL2-receptor is normally expressed and properly phosphorylated during cell activation in DS.

In addition to anti-CD3, several studies on proliferation of T-cells upon mitogenic stimulation and stimulation with recall antigens in DS have been performed. Use of patients of different ages combined with different mitogenic stimuli, such as phytohaemagglutinin (PHA), concanavalin (ConA), and pokeweed (PWM) make it difficult to compare most studies. Contrasting results are reported for responses upon stimulation with PHA in DS, being either normal [57;62-64] or significantly decreased. [47;58;65-68] Burgio has shown a clear effect of age, with normal response in children up to ten years of age and afterwards a significant decrease with increasing age. This is in contrast with Lockitch who has shown a small, not significant, increase of the response with age. Lower mitogenic responses are found in DS upon stimulation with PWM and ConA [58;67;68]. However, Lockitch et al have shown a reverse age effect with higher proliferation in DS than healthy controls upon PWM stimulation below six years of age but lower proliferation beyond six years. Stimulation with a recall antigen (Purified Protein Derivative, PPD) is followed by normal proliferation in DS. [68] A decreased proliferation in DS in response to the specific viral antigens of *Influenza A* and *B*, and also to tetanus toxoid has been described. [69;70] The decreased proliferation in response to *Influenza B* in DS is mainly due to a decreased response of CD4 T-cells. This effect is partially overcome in the presence of monocytes and B-cells.

PHA stimulation induces normal IL-2 production in DS. [65] Expression of IFNγ, encoded by chromosome 21, is found to be normal upon stimulation, but with a higher basal level. [71] No correlation with age is described. One study of serum levels of IFNγ in otherwise healthy patients with DS, aged 22-58 years, showed marked and significantly increased levels. [72] Epstein and Epstein have shown that stimulation of T-cells with PHA results in production of normal amounts of interferon in DS and normal proliferation. [64] The

addition of exogenous interferon to stimulation with PHA inhibits proliferation equally in both DS and controls. However, when ConA was used as stimulus, DS appeared to be significantly more sensitive to inhibition of proliferation by interferon. A reciprocal relationship was shown between stimulation with tetanus toxoid and the effect of interferon on this stimulation. Low levels of toxoid-stimulated proliferation results in increased stimulation by interferon in DS, while in controls at high levels, interferon has a more inhibitory effect on proliferation. It was suggested by Epstein et al. that this might be more the result of an effect of the toxoid than of the trisomic state. In addition, responses of different T-cell subsets may be involved in the differences in effect of the two mitogens used.

Antibody-dependent cell-mediated cytotoxicity (ADCC) is lower in children with DS compared to healthy controls. [37;47;67;73] Autologous mixed lymphocyte reaction (MLR) with T-cells and irradiated non-T-cells has been shown to be much lower in children with DS compared to healthy controls. [47] This result could not be confirmed by Gupta et al, who reported a normal response on autologous MLR in DS. [74] Age could be a possible explanation for this discrepancy in results, since the group studied by Franceschi et al was older. Allogeneic MLR resulted in either equal [47;74] or lower [36] activity in DS compared to controls. Dissociation between autologous and allogeneic MLR has been observed in auto-immune diseases, such as SLE, and normal aged individuals. $CD4^+$ T-cells are described to be the cells proliferating during MLR. The lower autologous MLR might therefore be explained by lower $CD4^+$ T-cell counts in DS.

Lymphocyte functional antigen-1 (LFA-1) is expressed on the cell surface of lymphocytes. LFA-1 is a heterodimeric molecule consisting of α (CD11a) and β chains (CD18), which is encoded on chromosome 21. Since LFA-1 has a role in intercellular adhesion, it has been suggested that over-expression of this antigen (due to a gene dosage effect in DS), would lead to increased aggregation of cells and subsequently causes cellular immune dysfunction. It has indeed been shown that LFA-1 is over expressed on lymphoid cells of DS. [75;76] However, the over expression could be explained by an abnormal distribution of lymphocyte subsets in DS that express different levels of LFA-1. [75;77] In addition, it was shown that DS in children under 2 years of age have increased levels of LFA-1, but consecutively lack an age-associated increase beyond the age of 2 in $CD4^+$, $CD8^+$, $CD45RO^-$ and $CD45RO^+$ subsets compared to controls resulting in comparable expression at an older age. [77] Although LFA-1 levels were comparable at an older age, significantly lower binding of T-cells to intercellular adhesion molecule 1 (ICAM-1) was shown in vitro in children with DS. This was also in apparent contrast to a previous study showing general increased adhesion of lymphocytes in DS. [77;78] A possible explanation for this defective binding by LFA-1 could be either abnormal T-cell activation or defective intracellular signal transduction in DS. These findings therefore do not support the

hypothesis of lymphocyte hyperadhesiveness as the cause of immunologic problems seen in DS.

An important function of T-cells is to regulate the immune response. Differentiation into $CD4^+$ T-helper (Th) subsets is an important step in selecting effectors functions. Cytokines are major contributors to a Th1 or Th2 type of response. While Th1 cytokines promote a cellular immune reaction, Th2 cytokines drive humoral immune responses. In children with DS no studies have been performed on this specific subject. However, a second type of T-cells with a regulatory function (Tregs, $CD25^+$ $FoxP3^+$ $CD4^+$ T-cells) has been reported in DS. One study described a relative increase of Tregs in DS, but no functional tests have been performed. [55]

In conclusion, different functional assays have been performed in DS, revealing decreased proliferation and cytotoxicity of T-cells in most of them. These combined studies lead to the hypothesis that accelerated thymic involution in children with DS results in both decreased numbers and dysfunction of T-cells.

B-cells

B-cell numbers

Children with DS were found to have decreased counts of B-lymphocytes compared to healthy children. [57;58] De Hingh et al. confirmed this and reported that the primary expansion of B lymphocytes as seen in healthy children in the first years of life does not occur in children with DS. [29] Throughout childhood, the B lymphocyte population remains severely decreased. It was suggested that this is caused by an intrinsic abnormality of the adaptive immune system. One of the possible causes is a decreased maturation of B-lymphocytes in DS. There is one early report however in 1975 by Burgio et al. that in 83 DS children the B cell counts were normal, compared to 76 controls. [63]

Overall, the number of B-cells is reported to be decreased.

B-cell function

Several studies have been done in children with DS in which serum levels of immunoglobulin A, M and G and/or IgG subclasses were measured. [56;58;63;79-82] Cocchi et al. showed normal IgA and IgG levels in children with DS [56], while in other studies elevated serum levels of IgA and IgG compared to controls were found. [58;63;79-82] This hyperglobulinemia might be explained by a slower elimination of infectious agents in DS, which may cause overstimulation of the immune system and overproduction of antibodies. IgM levels in DS are reported to be diminished [56;58;63] or normal. [79-82] In DS the IgG1 and IgG3 levels are often elevated, whereas the IgG2 and IgG4 levels are diminished. [79] Lower IgG2 and IgG4 levels may partially explain the increased susceptibility of children with DS to infections with encapsulated bacteria. In 1990 Anneren et al. reported an increase in serum

concentrations of IgG2 and IgG4 in DS after a selenium supplement of 10 microgram/kg/day during 6 months. [83] The parents reported spontaneously a reduced infection rate during this treatment. This study suggests that selenium might have an immunoregulatory effect. Costa-Carvalho et al. in 2006 evaluated the production of antibodies to a 23-valent pneumococcal vaccine in 17 children with DS (age 6 – 13 years). [80] Before the vaccination, these children had normal IgA and IgM levels, but they had elevated levels of total IgG, and the IgG subclasses of IgG1 and IgG3 and lower levels of IgG subclasses IgG2 and IgG4 than the controls. All DS children had a significant increase in the levels of antibodies (IgM and IgG2) to all serotypes of the vaccine, although these levels were lower than in the controls. Their advice is that this 23-valent pneumococcal vaccine could be of benefit in children with DS.

In conclusion, B-cell production of IgM antibodies is normal or decreased in children with DS, while IgG and IgA are normal or even increased.

EARLY SENESCENCE OF THE IMMUNE SYSTEM

The abnormal findings of thymic immunohistology and function in DS have lead to the concept of early senescence of the immune system in this specific population. Ageing not only results in thymic involution, but also in changes of telomere length and apoptotic rate of immune cells. Telomere shortening has a causal role in cellular ageing. With ageing shortening of telomere length is seen in lymphocytes. [84] In individuals with DS the rate of loss of telomeres of peripheral blood leukocytes is significantly increased compared to healthy controls. This loss of telomeric length is comparable in subpopulations of T-cells, B-cells and neutrophils of DS. Several explanations for the increased loss of telomeres in DS have been suggested; individuals with DS might have an increased cell division because of immunologic abnormalities. The rate of telomere loss could also be increased if the expression of genes involved in telomere length regulation is altered due to trisomy 21. Holmes et al. studied fetuses with DS to determine if accelerated telomere loss is associated with a stem cell deficiency, predisposing children with DS to clonal changes. [85] They found that leukocytes of fetuses with DS already showed significantly decreased telomere length. In addition, they showed that both fetuses and children with DS had significantly reduced numbers of hematopoietic stem cells, mostly from the myeloid lineage. In contrast, the erythroid progenitors were not affected in fetuses with DS.

The decreased numbers of immune cells seen in children with DS might be the result of increased loss due to apoptosis. In addition to increased apoptosis of granulocytes, Corsi et al. describe a higher proportion of $CD3^+$ T-cells in DS that express CD95, also known as the Fas-receptor that induces apoptosis upon Fas-ligand binding. [86] Purified

T-cells of children with DS showed a higher percentage of cells positive for Annexin-V compared to controls (33.2% versus 29.8%). However, cells positive for both propidium iodide and Annexin-V, indicative of necrotic cells, were decreased in DS (1.8% versus 3.7%). In addition, Roat et al studied apoptosis after in vitro treatment of PBMC with apoptogenic drugs. [87] Children with DS showed similar tendency to undergo apoptosis compared with controls, both unstimulated and after stimulation with apoptogenic drugs.

From these studies we conclude that in individuals with DS telomere shortening occurs already in fetal life, it continues after birth and in the myeloid lineage might be influenced by progenitor cell deficiency. With respect to apoptosis, an increased apoptotic rate probably does not play a role in the decreased numbers of immune cells in children with DS.

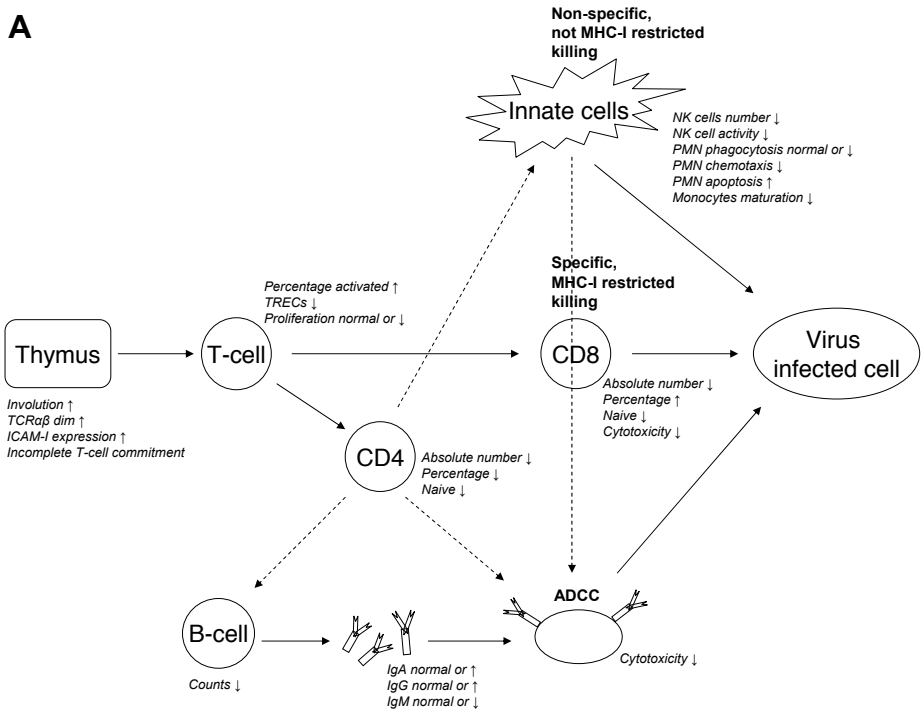

Figure 1. The role of immunologic abnormalities in pathogenic clearance in DS. *Continued on next page*

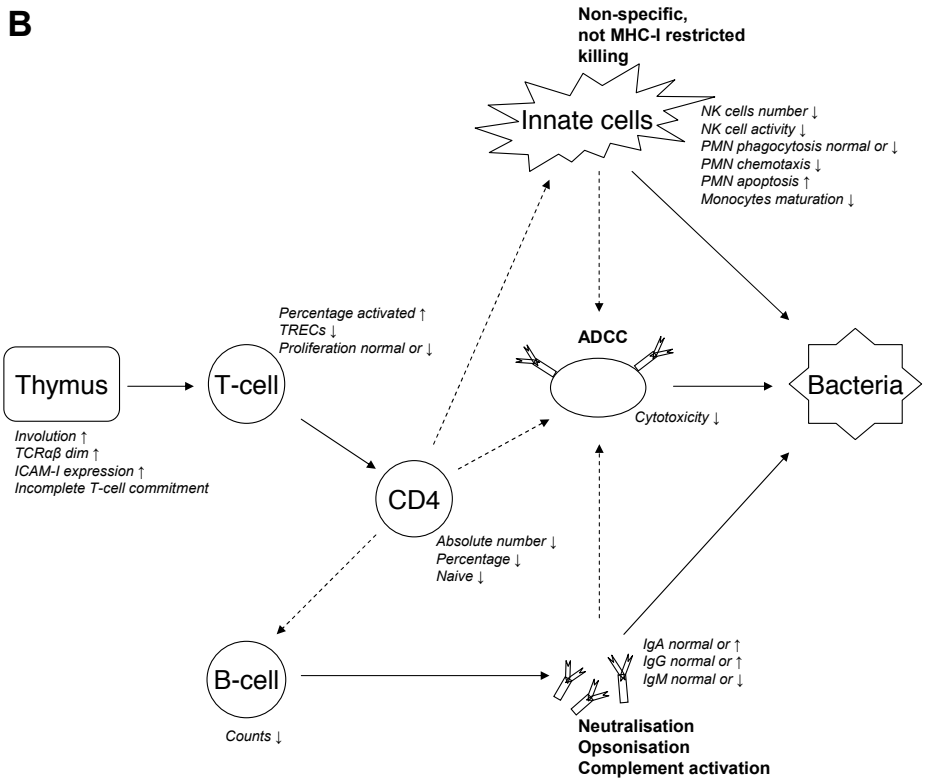

Figure 1. The role of immunologic abnormalities in pathogenic clearance in DS

The role of different immune cells in clearance of virus infected cells is shown in figure 1A. Viral immunity can be roughly divided in non-specific, not MHC-I restricted killing by innate cells (upper part of figure), specific, MHC-1 restricted killing by $CD8^+$ cytotoxic T-cells (middle part of figure) and antibody-dependent cell-mediated cytotoxicity (lower part of figure). Next to each cell type differences in number and function are summarized for children with DS. Figure 1B provides a summary of differences in cell types in DS that play a role in bacterial defense: not MHC-I restricted killing by innate cells (upper part of figure), antibody-dependent cell-mediated cytotoxicity (ADCC) (middle part of figure) and neutralisation, opsonisation and complement activation (lower part of figure).

IMMUNOLOGIC MECHANISMS UNDERLYING INCREASED INCIDENCE OF RESPIRATORY TRACT INFECTIONS IN DS

The previously described immunologic abnormalities in DS have been presented in diagrammatic form in figure 1 in an attempt to clarify the role of the immune system in the respiratory morbidity in children with DS. The first line defense against bacteria and viruses, the innate immunity, seems clearly disturbed in children with DS. Both quantitative and qualitative abnormalities have been shown for children with DS. Decreased numbers of NK cells, decreased NK-function, phagocytosis and chemotaxis of PMNs and

monocytes might result in decreased direct killing and clearance of pathogens in this specific population.

The next step in bacterial and viral defense, T-cell immunity, is impaired in DS as well. Accelerated involution of the thymus, abnormal thymic maturation and low thymic output might explain low absolute numbers of $CD4^+$ and $CD8^+$ T-cells, especially naïve T-cells, decreased cytotoxicity and peripheral proliferation in children with DS. Again, quantitative and qualitative defects in children with DS might result in decreased pathogenic killing and clearance. The increased percentage of activated T-cells described, might not play a role in pathogenic clearance, but rather reflect a chronic or auto-immunologic state in DS.

Besides T-cells, B-cell counts are lower in DS as well. Although IgM antibodies production is normal or decreased in DS, levels of other antibodies are normal or even increased (IgA, IgG). Whether this is caused by an intrinsic functional difference in B-cells or is a reflection of acute or chronic inflammation is unclear. Based on these numbers however, normal bacterial clearance would be expected in DS. On the other hand, despite even increased levels of IgG, ADCC is decreased in DS. Since ADCC depends not only on the presence of antibodies, but on NK-cell function as well, dysfunction of the latter might be causing the effect measured in DS.

Although increased telomeric loss has been shown in DS, one can question whether this might be the cause or consequence of bacterial and viral infections in DS. Since no evidence can be provided for a direct effect, we would suggest that it has no direct role in the susceptibility to RTIs in children with DS.

Future topics to be addressed

Although many previously performed studies suggest a role for an abnormal immune system in the pathofysiologic mechanism of increased susceptibility to RTIs in children with DS, definite evidence is still lacking. Future in vitro and in vivo immunologic investigations might give better insight in this issue. Several studies have suggested that low numbers and disturbed function of T-cells in children with DS are caused by abnormal thymic development and function. However, the establishment and maintenance of the naive T-cell pool is a dynamic process, also influenced by cellular lifespan and division. A study on naive T-cell dynamics incorporating thymic output, T-cell proliferation and T-cell loss by antigen driven differentiation or apoptosis would provide a more accurate answer to the role of thymic insufficiency in children with DS. Although multiple immunologic abnormalities have been described in individuals with DS, it is difficult to state whether these abnormalities are the cause or consequence of the high morbidity. Longitudinal studies are needed to determine the etiological relationship between the number of different immune cell types during early childhood in children with DS and the subsequent development of RTIs.

Since an abnormal immune system is suggested to be involved in the increased incidence of RTIs in children with DS, it is of outstanding interest to consider what preventive measures could be taken in this unique population. Unfortunately, the literature is insufficient to point out specific pathogens as major cause of the respiratory morbidity in children with DS. It is unclear what pathogens, either viral or bacterial, have an increased contribution in this matter. Therefore, it is difficult to state if antibiotic prophylaxis or specific immunoglobulines would be of use in children with DS. Even if certain pathogens can be defined as a risk factor for the development of severe RTIs, preventive measurements are a matter of debate. For example, the finding of DS as a new risk factor of RSV associated hospitalization has led to discussions on the role of passive immunization in this specific group of children. Although passive immunization against RSV has been approved for certain risk groups, such as premature born children and children with significant congenital heart disease, it is questionable if it is effective in children with DS as well. And if so, it is questionable if health gain is achieved at acceptable costs. Based on the long-term implications for children with DS and society, an international multi-centre randomized-controlled clinical trial on the use of passive immunization in children with DS is required before any recommendations can be given.

CONCLUSIONS

Children with DS have an increased incidence of RTIs which might be associated with congenital heart disease, abnormal airway anatomy and physiology, hypotonia, and aspiration. In addition, patients with DS show multiple abnormalities both in numbers and function of both innate and adaptive immunity. These immunologic abnormalities combined, whether or not directly interacting with each other, strongly suggest diminished viral and bacterial clearance in DS. Although it can be suspected that certain findings reflect a state of inflammation in DS rather than being the cause, we believe that the high incidence of RTIs in children with DS is the consequence of an impaired immune system. Future studies on T-cell dynamics and the etiological relationship between numbers of different immune cell types during early childhood and the subsequent development of RTIs might provide better insight into this hypothesis.

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Chapter 3

Distinct abnormalities in the innate immune system of children with Down syndrome

Beatrijs L. P. Bloemers
Grada M. van Bleek
Jan L. L. Kimpen
Louis Bont

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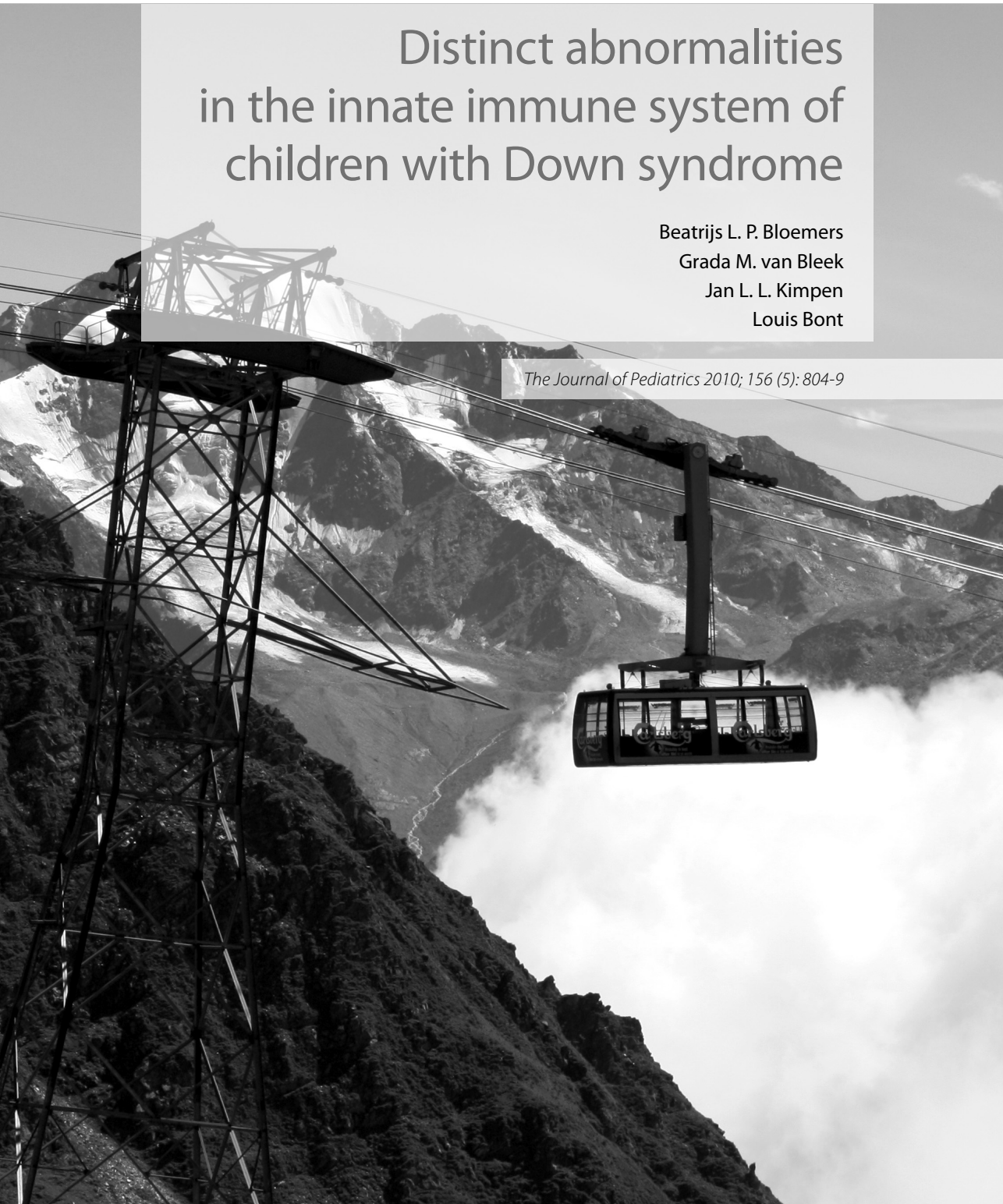

ABSTRACT

Objectives

To analyze the frequency and phenotype of cells of the innate immune system in the peripheral blood of children with Down syndrome (DS).

Methods

Flow cytometric analysis of expression of cell surface markers was performed in children with DS (n=41) and healthy age-matched controls (n=41).

Results

Compared with controls, children with DS had significantly lower absolute total leukocyte counts, lymphocytes, monocytes and granulocytes, but 1.5-times higher absolute numbers of $CD14^{dim}CD16^{+}$ monocytes (147 x 10^6/l vs 93 x 10^6/l; p=0.02). This difference is fully explained by a higher percentage of $CD14^{dim}CD16^{+}$ monocytes within the monocyte compartment (28.7% vs 13.4%; p<0.001). The absolute numbers of myeloid dendritic cells were lower in DS (13.8 x 10^6/l vs 22.7 x 10^6/l, p<0.001). The numbers of plasmacytoid dendritic cells and natural killer cells were normal. Absolute numbers of invariant natural killer T-cells were very low overall, but significantly lower in children with DS than in controls (1.2 x 10^6/l vs 3.7 x 10^6/l, p=0.01).

Conclusions

Children with DS exhibited distinct abnormalities in cells of the innate immune system. Most strikingly, they had a high number of proinflammatory $CD14^{dim}CD16^{+}$ monocytes. This elevated level of $CD14^{dim}CD16^{+}$ monocytes may play an important role in the onset and maintenance of chronic inflammatory disease in DS.

INTRODUCTION

Down syndrome (DS) is the most common chromosomal abnormality among live-born infants, with an incidence of 1 in 625 in the Netherlands. [1] DS is characterized by various dysmorphic features and congenital malformations, including congenital heart disease and gastrointestinal disease, as well as increased respiratory morbidity. [2;3] DS also is associated with various immunologic impairments. Hematologic malignancies and autoimmune diseases, such as hypothyroidism, celiac disease, and diabetes mellitus, are more prevalent in children with DS. [4-8] Children with DS seem to have an altered adaptive immune response, ascribed to abnormal thymus development and function. [9-13] B-cell and T-cell numbers are low, especially in the first 2 years of life. [14] In addition, the percentage of naïve T-cells is lower than that in children without DS. [13;15] Studies on the effect of age on lymphocyte counts have demonstrated an inverse correlation. [14;16;17] Within the innate immune system, only natural killer (NK) cells have been studied in children with DS. Lower absolute numbers of $CD16^{+}CD56^{+}$ cells have been found [14;18]. A study of 28 children with DS and 13 nonmatched controls found decreased NK-cell cytotoxic activity in DS. [18] The present study investigated the age-specific frequency of cell lineages of the innate immune system in the peripheral blood of children with DS.

METHODS

Study population

The study group comprised 41 children with DS, age 0-12 years, from the Wilhelmina Children's Hospital Utrecht, The Netherlands. The children were divided into 6 different age groups: 0 to 2 years, 2 to 4 years, 4 to 6 years, 6 to 8 years, 8 to 10 years, and 10 to 12 years. Because the greatest developmental changes in the immune system occur during approximately the first 2 years of life, the 0- to 2-year age group included more than 3 times more children (n=16) than the other age groups, each of which included 5 children. [19;20] Children with a history of surgery in the previous 2 months were excluded. An equal number of age-matched healthy children admitted to the hospital for elective urologic, plastic, ophthalmologic, or general surgery were included as controls. In these children blood was drawn before or directly after introduction of anesthesia, to minimize immunologic interference. Some children included in the study had a history of recurrent infections of either the urinary tract (7 controls) or respiratory tract (5 DS, 1 control) and received prophylactic antibiotics during the study. No children had any signs of infection in the week before or at the time of blood collection. Parental written informed consent was obtained for all patients. The research protocol was approved by the University Medical Centre Utrecht's Medical Ethics Committee.

Absolute leukocyte differentiation

Absolute leukocyte count and differentiation was determined using multiangle polarized scatter separation (MAPSS) plus 3-color fluorescent on a CELL-DYN Sapphire analyzer (Abbott Diagnostics, Abbott Park, Illinois)

Cell surface immunophenotyping

Blood was collected in EDTA-treated tubes for B-cell immunophenotyping and in sodium heparin- containing tubes for all other cell lineages. Whole blood was stained using a standardized protocol based on manufacturer's instruction with the following monoclonal antibodies: fluorescein isothicyanate-conjugated anti-CD3, -CD4, -CD8, -CD14, -CD45RA, –Lineage 1, -IgM, -IgG, -IgD, and -CD38; phycoerythrin-conjugated anti-CD16, -CD27, -CD38, -CD123, -CCR7, -IgD, -IgA, and -CD10; peridin-chlorophyll protein-conjugated anti-CD4, -CD8, -CD28, -HLA-DR, and -CD19; and allophycocyanin-conjugated anti-CD3, -CD11c, -CD45R0, -CD56, -CD27, and -CD38 (all from BD Biosciences, San Jose, California). All samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences). For each sample, a minimum of 10 000 lymphocytes were collected, except for B-cells (80 000 cells) and invariant NK T (iNKT) cells (100 000 cells). Data analysis was performed with CellQuest software (BD Biosciences).

Population definitions

The following cells of the innate immune system were studied: classical $CD14^{+}CD16^{-}$ and non-classical $CD14^{dim}CD16^{+}$ monocytes, plasmacytoid dendritic cells (pDCs; $CD123^{+}$ $CD11c^{-}$), myeloid dendritic cells (mDCs; $CD123^{dim}CD11c^{+}$), NK cells ($CD3^{-}CD16^{+}CD56^{+}$), and iNKT cells ($V\alpha24^{+}V\beta11^{+}$). In addition, subclasses of T- and B-cells were studied. Within CD4 and CD8 T-cells naïve ($CCR7^{+}CD45R0^{-}$), central memory ($CCR7^{+}CD45R0^{+}$), effector memory ($CCR7^{-}CD45R0^{+}$) and terminally-differentiated end-stage effector ($CCR7^{-}$ $CD45R0^{-}$) subsets were identified. B-cell subsets were defined as pre-B-cells ($CD38^{+}IgD^{-}CD10^{+}$), recent bonemarrow emigrants ($CD38^{+}IgD^{+}$ $CD10^{+}$), naïve B-cells ($CD38^{-}IgD^{+}CD27^{-}$), activated B-cells ($CD38^{+}IgD^{-}CD10^{-}$) and memory IgM ($IgM^{+}IgD^{+}CD27^{+}$), IgG ($IgG^{+}IgA^{-}$ $CD27^{+}$), and IgA ($IgG^{-}IgA^{+}CD27^{+}$) B-cells.

Statistical analysis

Differences in baseline characteristics were compared by the χ^2 test. Values are expressed as mean ± standard error of the mean. Regression analysis including sex and age was performed to identify differences between children with DS and healthy controls. All statistical analyses were performed using SPSS for Windows version 12.0.2 (SPSS Inc., Chicago, Illinois). A *P* value of .05 was considered the limit of significance.

RESULTS

Study population

Forty-one non-institutionalized children with DS (22 males) were compared to 41 children without DS (28 males) ranging in age from 0 to 12 years (median age 4.7 versus 3.7 years). The subjects' clinical characteristics are summarized in Table 1. Leukocyte differentiation showed significantly lower absolute counts in total leukocytes, total lymphocytes, $CD4^+$ T-cells, $CD19^+$ B-cells, monocytes and granulocytes (Figure 1).

Distinct abnormalities in the innate immune system

Table 2 summarizes the main results of this study. As shown in Figure 1, A, absolute monocyte counts were significantly lower in children with DS (504 x 10^6/l versus 651

Table 1. Baseline characteristics

	DS	HC
Total patients	41	41
Male	22	28
Median age, years (IQR)	4.7 (0.9-9.3)	3.7 (1.0-8.4)
Age < 2 years, number	16	15
Age > 2 years, number	25	26
Congenital heart disease	18	-
Hemodynamically significant	3	-
Status postsurgical correction	10	-
Hemodynamically insignificant	5	-

IQR indicates interquartile range.

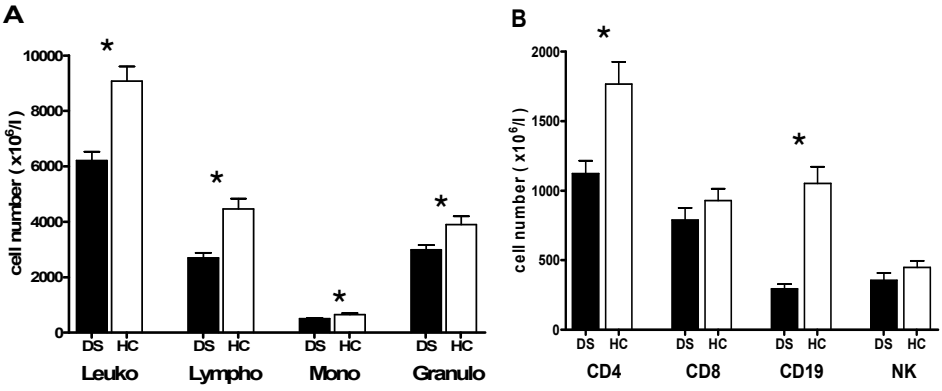

Figure 1. Leukocyte populations

Absolute numbers of leukocyte populations are shown. * $p<0.01$ for children with DS (*black bars*) compared with healthy controls (HC; *open bars*).

Leuko, leukocytes; lympho, lymphocytes; mono, monocytes; granulo, granulocytes; NK, natural killer cells.

Table 2. Absolute and relative numbers of innate immunity cells

		DS*	HC*	P-value
Monocytes				
$CD14^{dim}CD16^{+}$	x 10^6/l	**147 (18)**	93 (13)	0.02
	%	**28.7 (2.4)**	13.4 (1.3)	<0.001
$CD14^{+}CD16^{-}$	x 10^6/l	357 (31)	**558 (37)**	<0.001
	%	71.3 (2.4)	**86.6 (1.3)**	<0.001
pDCs ($CD123^{+}CD11c^{-}$)	x 10^6/l	15.0 (2.5)	18.7 (1.8)	NS
	%	30.8 (2.4)	30.1 (1.7)	NS
mDCs ($CD123^{dim}CD11c^{+}$)	x 10^6/l	13.8 (1.2)	**22.7 (1.9)**	<0.001
	%	31.1 (2.3)	**37.2 (2.2)**	0.05
NK ($CD16^{+}CD56^{+}$)	x 10^6/l	356 (52)	447 (48)	NS
	%	9.9 (0.9)	8.3 (0.7)	NS
iNKT ($V\alpha24^{+}V\beta11^{+}$)	x 10^6/l	1.2 (0.2)	**3.7 (0.9)**	0.01
	%	0.04 (0.01)	0.1 (0.03)	NS

NS indicates not significant. In bold the significantly increased number or percentage of cells compared to the other group is given.

* Mean absolute and relative cell counts are shown with standard error of the mean in parentheses.

x 10^6/l; p<0.01). An example of flow cytometric analysis of monocyte subsets is provided in Figure 2. A remarkable finding was the increased percentage of $CD14^{dim}CD16^{+}$ monocytes in children with DS at all ages (p<0.001) (Figure 3, B). Absolute numbers of $CD14^{dim}CD16^{+}$ monocytes were 1.5 times higher in children with DS compared with

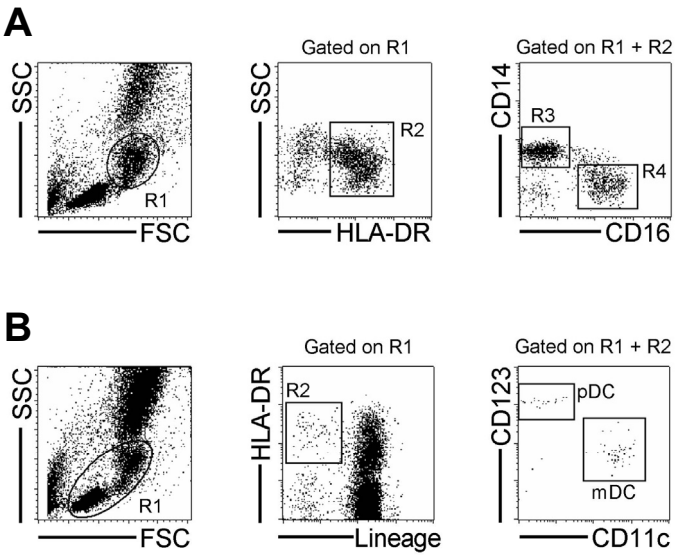

Figure 2. Flow cytometric analysis

An example of flow cytometric analysis of monocyte subsets (A) and dendritic cells (B) is shown. Monocyte subsets, gated on monocytes (R1) and HLA-DR-positive cells (R2) are shown. Percentages of classical $CD14^{+}CD16^{-}$ (R3) and non-classical $CD14^{dim}CD16^{+}$ (R4) subsets were calculated within total monocyte numbers. Plasmacytoid and myeloid dendritic cells were gated on the monocyte-lymphocyte population (R1) and $lineage^{-}HLA\text{-}DR^{+}$ cells (R2).

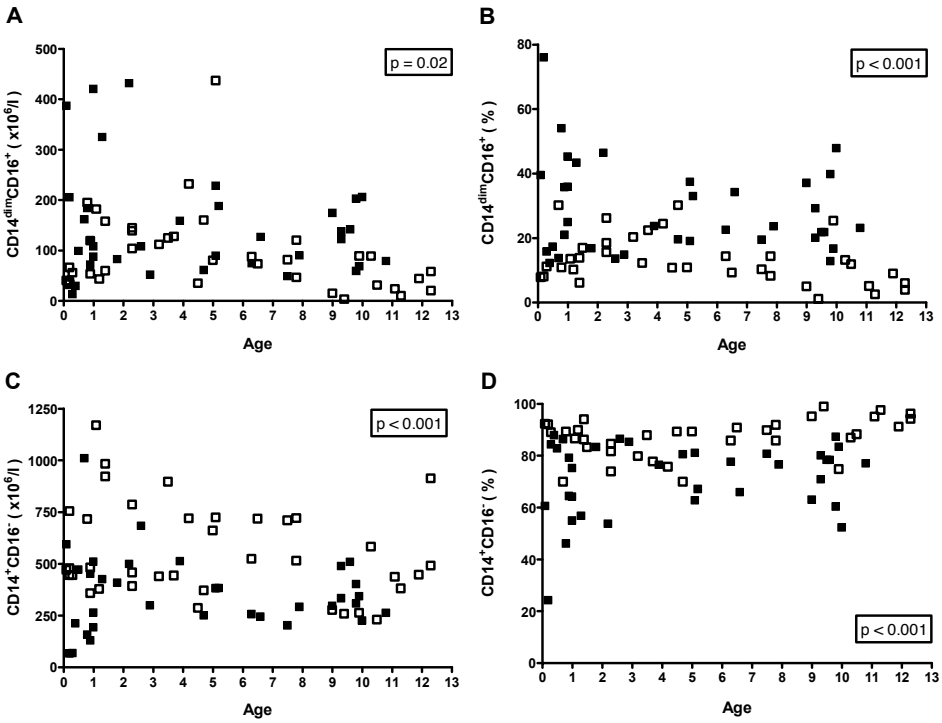

Figure 3. Monocytes

Age distribution of absolute (A, C) and relative (B, D) numbers of non-classical $CD14^{dim}CD16^{+}$ (A, B) and classical $CD14^{+}CD16^{-}$ (C, D) monocyte subsets in children with DS (*black squares*) and healthy controls (*open squares*).

healthy controls (147 x 10^6/l versus 93 x 10^6/l; p=0.02) (Figure 3, A). This comparison was repeated using the Mann-Whitney *U* test, because the distribution was not fully normal. The sensitivity analysis confirmed a higher median $CD14^{dim}CD16^{+}$ monocyte count in children with DS (119 x 10^6/l vs 76 x 10^6/l; p=0.006; data not shown). Consequently, absolute $CD14^{+}CD16^{-}$ monocyte counts and percentages were decreased in children with DS (p<0.001 for both) (Figure 3, C and D). Regression analysis confirmed that differences between DS and controls were independent of sex and age.

Figure 2, B shows the results of flow cytometric analysis of dendritic cells. The absolute counts and percentages of pDCs were comparable in children with and without DS (Figure 4, A and B). Children with DS had significantly lower absolute mDC counts compared with controls (Figure 4, C). In the 0- to 2-year age group, the percentage of mDCs was 2 times lower in children with DS compared with controls (17.8% vs 32.6%; p=0.01) (Figure 4, D and E). After age 2 years, this percentage was similar in the 2 groups.

Absolute NK-cell counts were lower in children with DS compared with controls in all age groups, but the difference did not reach statistical significance (Figure 5, A). In chil-

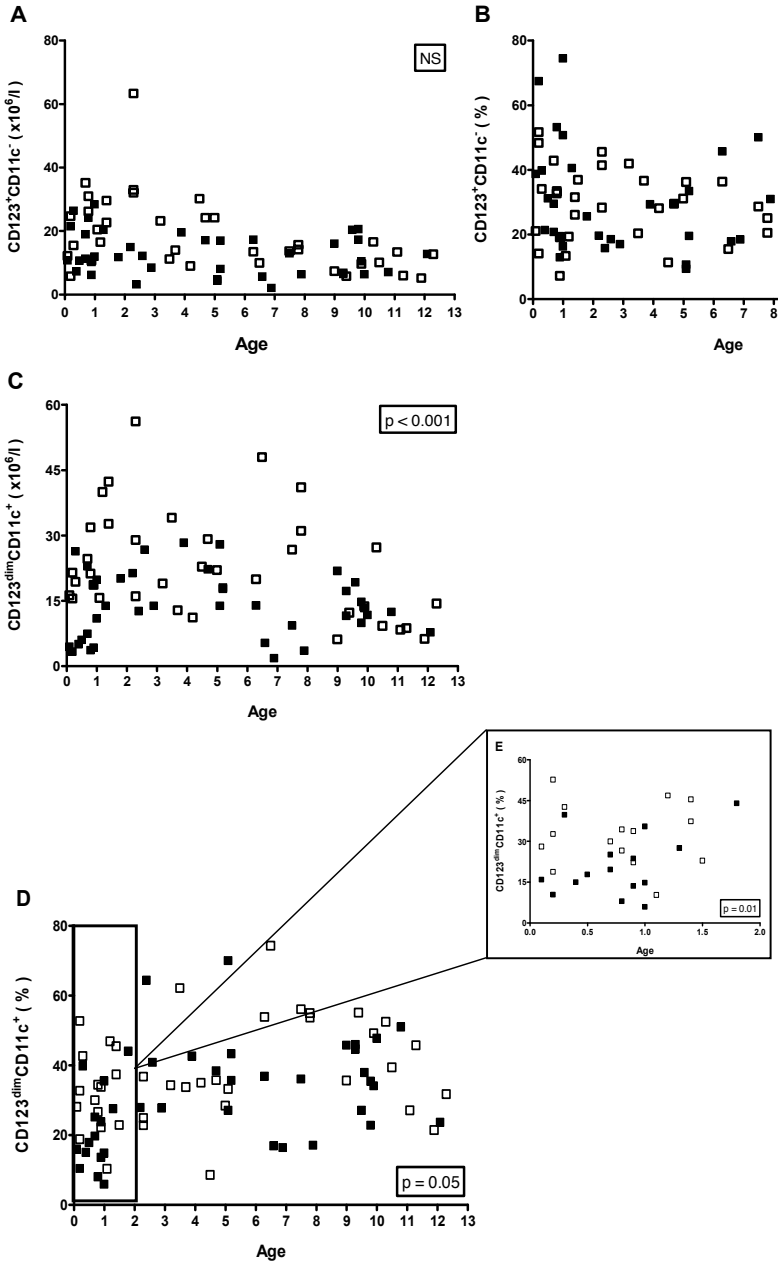

Figure 4. DCs

Age distribution of absolute (A, C) and relative (B, D) numbers of $CD123^{+}CD11c^{-}$ pDCs (A, B) and $CD123^{dim}CD11c^{+}$ mDCs (C, D) in children with DS (*black squares*) and healthy controls (*open squares*). Subanalysis under age 2 years revealed significantly lower percentages of $CD123^{dim}CD11c^{+}$ dendritic cells in children with DS compared with healthy controls (E).

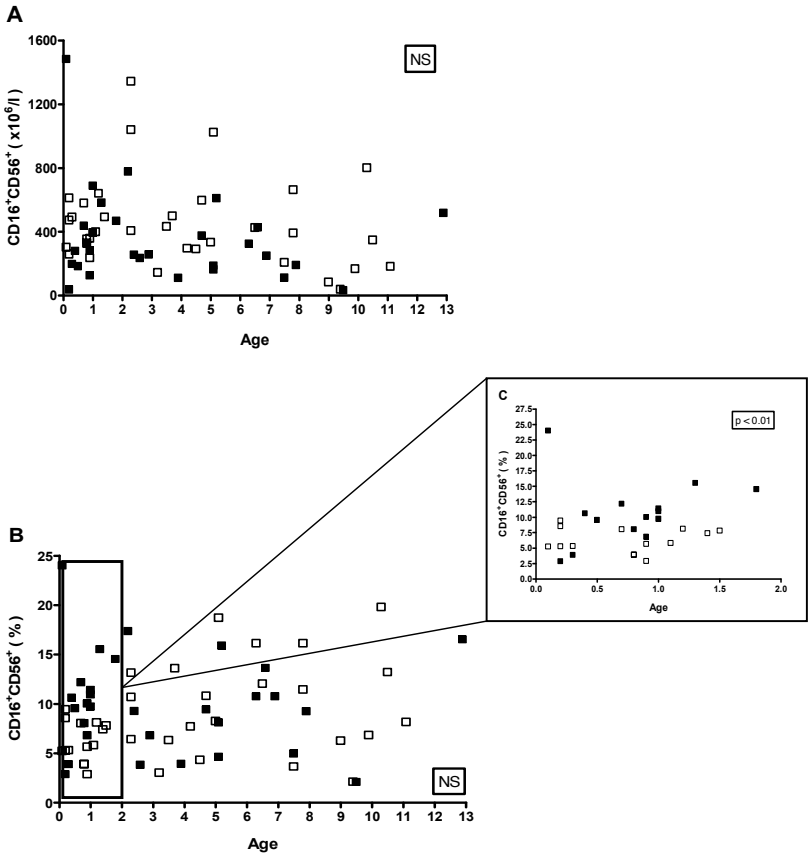

Figure 5. NK-cells

Age distribution of absolute (A) and relative (B) numbers of $CD16^+CD56^+$ NK-cells. Subanalysis under age 2 years revealed significantly increased percentages of $CD16^+CD56^+$ NK-cells in children with DS (*black squares*) compared with healthy controls (*open squares*) (C).

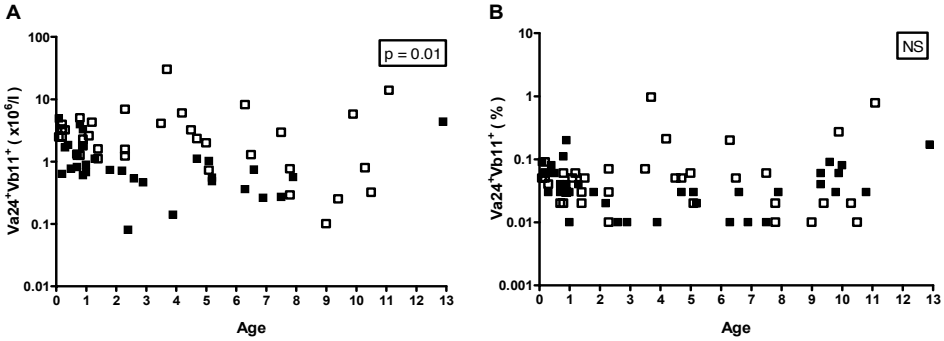

Figure 6. Invariant NKT-cells

Age distribution of absolute (A) and relative (B) numbers of $V\alpha24^+V\beta11^+$ iNKT-cells in children with DS (*black squares*) compared to healthy controls (*open squares*).

dren age <2 years, the percentage of NK-cells was significantly higher in the DS group compared with controls (10.3% vs 5.7%, p<0.01) (Figure 5, B and C).

As expected, iNKT ($V\alpha24^+V\beta11^+$) cell counts were very low in children with and without DS. At all ages, absolute iNKT cell counts were 3 times lower in children with DS compared with controls (1.2 x 10^6/l vs 3.7 x 10^6/l, p=0.01) (Figure 6, A). There was no effect of age on iNKT cell count. No significant between-group difference in percentage of iNKT cells was seen (Figure 6, B).

Distinct abnormalities in the adaptive immune system

Lower percentages of naïve T-cells within both CD4 and CD8 T-cell compartments were identified, confirming existent data in the literature (Figure 7, A and C). [13;15] Our results demonstrate for the first time that this difference is consistent at all ages (Figure 7, B and D). An inverse correlation between percentages of naïve CD4 and CD8 T-cells with age was found in both children with DS and controls. No major abnormalities in B-cell dif-

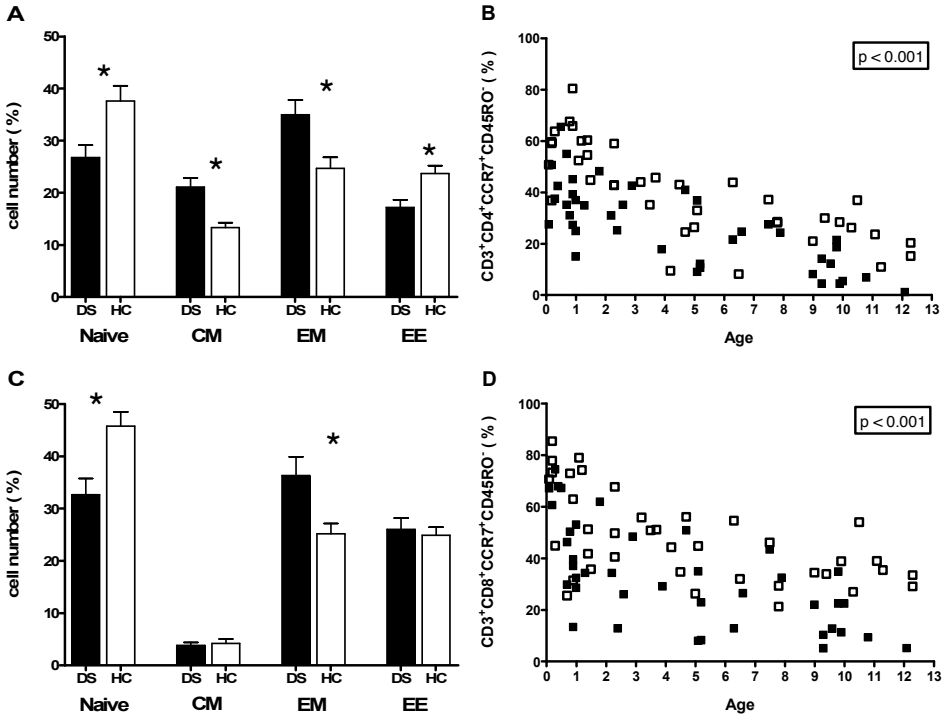

Figure 7. T-cell subsets

Relative numbers of CD4 (A, B) and CD8 (C, D) T-cell subsets are depicted. Age distribution of naïve subsets ($CCR7^+CD45R0^-$) is shown for both CD4 (B) and CD8 (D) T-cells in children with DS (*black squares*) compared to healthy controls (HC; *open squares*).

* p<0.005 for all $CD4^+$ T-cell subsets; p<0.001 for naïve $CD8^+$ T-cells; p<0.02 for effector memory $CD8^+$ T-cells. CM, central memory ; EM, effector memory; EE, end-stage effector.

ferentiation were found; however, the percentage of activated $CD38^{+}IgD^{-}CD10^{-}$ B-cells was higher in children with DS compared with controls (3.5% vs 1.1%, $p<0.001$) (Figure 8), with no effect of age. In addition, small differences in B-cell subset distribution were found; for example, the percentage of IgG memory B-cells was lower in children with DS (3.5% vs 5.5%, $p<0.05$). Regression analysis confirmed that the effect of DS on the frequency of B-cells and T-cells was independent of sex and age.

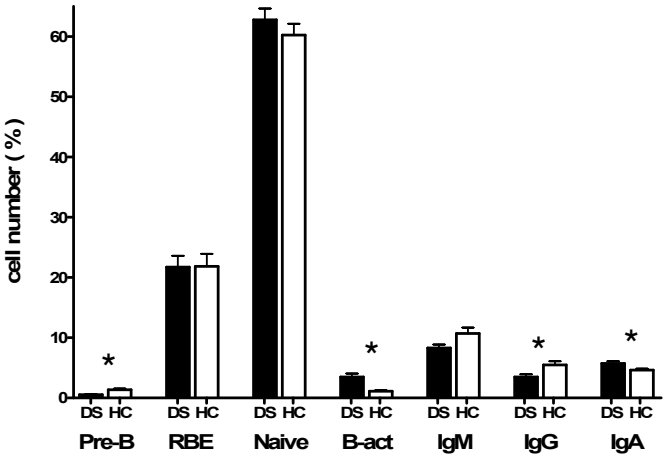

Figure 8. B-cell subsets

Relative numbers of $CD19^{+}$ B-cells are shown for children with DS (*black bars*) compared with healthy controls (HC; *open bars*). * $p<0.001$ for Pre-B-cells and activated B-cells, $p<0.05$ for memory IgG and IgA B-cells

Pre-B, pre-B-cells ($CD38^{+}IgD^{-}CD10^{+}$); RBE, recent bone marrow emigrants ($CD38^{+}IgD^{+}CD10^{+}$); Naïve, naïve B-cells ($CD38^{-}IgD^{+}CD27^{-}$); B-act, activated B-cells ($CD38^{+}IgD^{-}CD10^{-}$); IgM, memory IgM B-cells ($IgM^{+}IgD^{+}CD27^{+}$); IgG, memory IgG B-cells ($IgG^{+}IgA^{-}CD27^{+}$); IgA, memory IgA B-cells ($IgG^{-}IgA^{+}CD27^{+}$).

DISCUSSION

This study has investigated for the first time in detail, the frequency of cells of the innate immune system in peripheral blood in children with DS. In a cohort of 41 children with DS and an equal number of age-matched healthy controls, we found overall lower absolute counts of all main leukocyte lineages. We detected distinct abnormalities in the innate immune cells of children with DS, with higher numbers of $CD14^{dim}CD16^{+}$ monocytes, but lower numbers of mDCs and iNKT cells. We also confirmed previous reports of lower percentages of naïve $CD4^{+}$ and $CD8^{+}$ T-cells in children with DS. [13;15]

We found high percentages and absolute numbers of $CD14^{dim}CD16^{+}$ monocytes. Human blood monocytes can be divided into 2 subpopulations based on CD16 expression. 21 The majority of human blood monocytes, known as classical monocytes, are strongly

positive for the CD14 cell surface molecule, but do not coexpress CD16. In contrast, about 10% coexpress the FcyRIII (CD16) molecule, and are referred to as non-classical or "proinflammatory" monocytes. It has been suggested that non-classical $CD14^{dim}CD16^{+}$ monocytes were derived from classical $CD14^{+}CD16^{-}$ monocytes but developed into a more mature and more active cell type. [22] In addition, $CD14^{dim}CD16^{+}$ monocytes have superior antigen-presenting cell activity, produce higher levels of proinflammatory cytokines (eg, tumor necrosis factor and interleukin-10) and have direct antibacterial activity in the tissue. [23-26] In contrast, $CD14^{+}CD16^{-}$ monocytes have a scavenging function in the blood, removing apoptotic neutrophils and debris. The true functions of both subsets of human blood monocytes under physiological or pathological conditions remain incompletely understood, however. Various acute and chronic inflammatory diseases, including sepsis, malignancies and human immunodeficiency virus infection, have been associated with increased numbers of $CD14^{dim}CD16^{+}$ monocytes in the peripheral blood. [26-29] The heterogeneity of these diseases suggests that the induction of $CD14^{dim}CD16^{+}$ monocytes is not the cause of disease inception, but rather is a non-specific consequence of (chronic) inflammation. Our results indicate a high frequency of $CD14^{dim}CD16^{+}$ monocytes in very young children with DS who had no clinical signs of acute or chronic inflammatory disease. One possible explanation for this finding is a chronic, clinically covert inflammation in DS. Alternatively, this finding might indicate that these cells actually have a role in the susceptibility to autoimmune diseases associated with chronic inflammation in DS later in life. Longitudinal cohort studies are needed to distinguish between these two explanations of the elevated number of $CD14^{dim}CD16^{+}$ monocytes in children with DS.

A second distinct abnormality was our finding of decreased iNKT cells in DS. iNKT cells are a specialized subset of thymus-derived T-cells characterized by the expression of an invariant T-cell receptor (Vα24 and Vβ11). [30] Children with DS are known to have accelerated thymic involution. Our findings confirm previous reports of decreased T-cells in DS, which has been hypothesized to be due to thymic involution, resulting in decreased thymic output. Our finding of decreased absolute numbers of iNKT cells might be similarly explained by decreased thymic output of these cells. Future functional studies of iNKT cells are needed to explore whether these cells have intrinsic defects that also could affect the susceptibility to viral infections and autoimmunity in DS.

A third distinct abnormality of the innate immunity was our finding of lower numbers of mDCs in DS, especially in the first 2 years of life. mDCs are professional antigen-presenting cells that promote a Th1-type response of CD4 T-cells during viral infection. [31;32] Low mDC numbers in the peripheral blood may be associated with abnormalities in T-cell maturation in DS. More functional studies on dendritic cell-T-cell interactions in children with DS are needed to explore this hypothesis further.

Different mechanisms may underlie the distinct abnormalities in innate immunity cells in the peripheral blood in DS. First, it has been postulated that early immunologic senescence explains the clinical and immunologic abnormalities in patients with DS. Healthy ageing of the innate immune system is accompanied by an increase in the numbers of circulating monocytes and NK-cells and a decrease in the number of mDCs and iNKT cells. [33] Although absolute iNKT counts were decreased in DS, no difference in percentage was found, and no effect of age was identified. An increase in mDC numbers, not a decline, was seen with age. The numbers of both monocytes and NK-cells did not exhibit a positive correlation with age. Thus, our findings do not support precocious aging as an explanation for the innate immune system abnormalities found in our DS patients.

Second, bone marrow dysfunction may be considered. This seems unlikely, however, because some cell types are present in normal or even increased numbers in the blood of children with DS. The possibility of more complex dysregulation of bone marrow function cannot be excluded.

Third, abnormal maturation and differentiation of the monocyte population may be considered to explain our findings of increased numbers of $CD14^{dim}CD16^{+}$ monocytes and decreased mDCs.

Fourth, as mentioned earlier, it is conceivable that our findings are secondary to chronic inflammation in DS, because many of our findings are immunologic features of chronic inflammation. Future studies are warranted to examine whether abnormal cell counts in the innate immune system are due to intrinsic abnormalities in cellular differentiation or the consequence of chronic inflammation.

This study has some potential methodological limitations. First, the small numbers of patients and controls make this study underpowered to detect differences in some subsets. For example, we could not confirm previous results showing lower NK-cell numbers in children with DS, although a trend was evident. Second, technical problems reduced the group size in NK and iNKT cells. Third, the control group of children with DS comprised children admitted for elective surgery. Although blood samples were collected before or immediately after anesthesia was administered, we cannot exclude the possibility of immunologic interference by the anesthetic agents. Finally, our studies in the peripheral blood of children with DS do not allow us to draw any conclusions regarding immune responses in the tissue, where most inflammatory diseases occur.

Our results demonstrate distinct abnormalities in cells of the innate immune system of children with DS. These abnormalities might play a role in these children's increased susceptibility to viral infections, hematologic malignancies, and autoimmune diseases. Whether certain findings, particularly the high frequency of $CD14^{dim}CD16^{+}$ monocytes, are intrinsic to children with DS or are the consequence of chronic inflammation remains to be elucidated.

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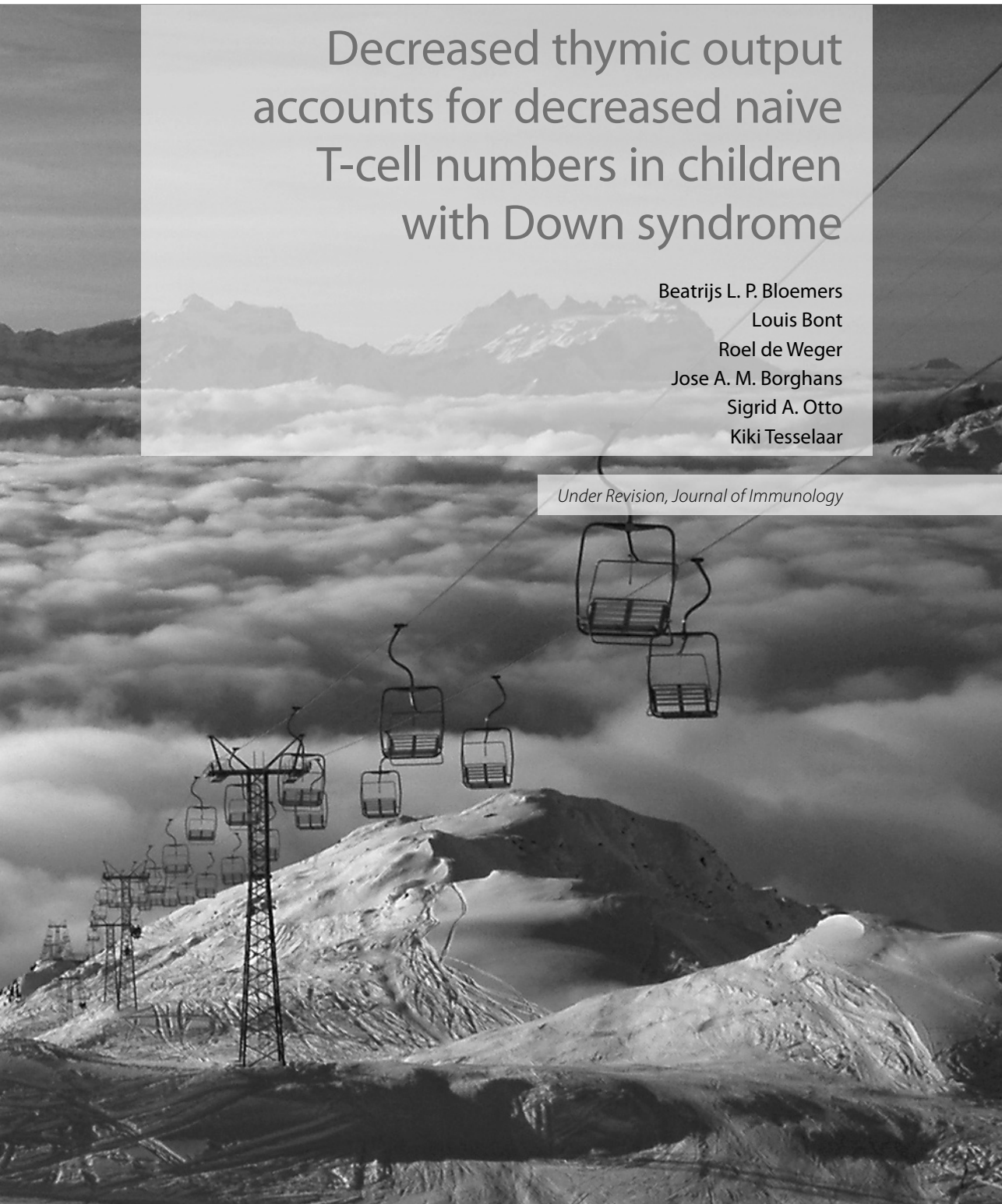
Chapter 4



Decreased thymic output accounts for decreased naive T-cell numbers in children with Down syndrome

Beatrijs L. P. Bloemers
Louis Bont
Roel de Weger
Jose A. M. Borghans
Sigrid A. Otto
Kiki Tesselaar

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ABSTRACT

Children with Down syndrome (DS) have low numbers of naive T-cells and abnormal thymus development and function. Since next to thymic production, peripheral proliferation greatly contributes to naive T-cell generation in healthy children, we examined the cause of reduced naive T-cell numbers in children with DS. Compared to aged matched controls, the total number of signal joint T-cell receptor excision circles (sjTREC) per ml blood was reduced in DS. Reduced frequencies and absolute numbers of protein tyrosine kinase 7 (PTK7) positive recent thymic emigrants, but similar levels of naive T-cell apoptosis and antigen-driven activation in DS suggested that reduced thymic output and not increased peripheral loss of naive T-cells caused the reduced sjTREC numbers. We found no support for defective peripheral generation of naive T-cells in DS. In DS the naive T-cells responded to IL-7 and, based on Ki-67 expression, had similar proliferation rates as in healthy controls. sjTREC content per naive $CD8^+$ T-cells were not increased but even decreased, pointing to increased survival or peripheral generation of naive T-cells in DS. In conclusion we here show that reduced thymic output, but not reduced peripheral generation nor increased loss of naive T-cells result in the low naive T-cells numbers found in DS.

INTRODUCTION

The most common chromosomal abnormality of live-born infants is Down syndrome (DS). Children with DS have a high morbidity because of respiratory tract infections. [1,2] In addition, various immunological impairments are associated with DS. Children with DS have a high incidence of hematologic malignancies and auto-immune diseases like hypothyroidism, celiac disease and diabetes mellitus. [3-7]

Studies on the adaptive immune system of DS have shown that total lymphocyte numbers are decreased in this population. [8-14] This holds true for the $CD4^+$ and $CD8^+$ T-cell subsets, especially in the first two years of life, but becomes less pronounced with increasing age and eventually normalizes around the age of 16 years. Subdivision of T-cells into naive and memory cells showed that in fact only the naive subsets are smaller compared to healthy controls. [8,9,11-13] In contrast, the fraction of $CD8^+$ T-cells within the T-cell compartment is increased and the fraction of $CD4^+$ T-cells decreased. [9] Percentages of naive cells have been described to be decreased and memory and effector subsets to be increased in both $CD4^+$ and $CD8^+$ T-cells. [15-17] Several studies have ascribed the immunologic impairment of T-cells in DS to abnormal thymus development and function. [16,18-21] Children with DS show increased cortical thymocyte depletion, cystic changes and fibrosis, suggestive of an accelerated involution of the thymus as seen in elderly. Although thymocyte maturation appears disturbed, normal mature T-cells expressing CD3 and TCRαβ are found in peripheral blood. [21,22]

Establishment and maintenance of the peripheral naive T-cell compartment are dynamic processes. T-cells are produced by the thymus and are released in peripheral blood as recent thymic emigrants (RTE). Since these cells are the most proximal to the thymus, they are essential for maintaining a diverse $\alpha\beta$ TCR repertoire. This proximity is also reflected by their high content of signal joint TCR gene excision circles (sjTRECS), which are circular DNA products of intrathymic V(D)J recombination. Peripheral proliferation of naive T-cells and longevity are the other mechanisms contributing to establishment and maintenance of the naive T-cell pool. Main drivers of these processes are IL-7, [23] and TCR-MHC/selfpeptide ligand interactions. [24,25] Based on phenotypic markers several naive $CD4^+$ T-cell subsets with different dynamic histories can be distinguished. [24] Recently, PTK7 has been described as a novel marker for $CD4^+$ RTE. [26] Naive CD31 positive and PTK7 positive $CD4^+$ T-cells are considered to be the subset most proximal to the thymus and MHC /selfpeptide ligand induced proliferation of these cells has been suggested to lead to loss of PTK7 and subsequently CD31 expression. IL-7 preferentially induces proliferation of the CD31 positive subset thereby down modulating CD127 expression but not inducing any other phenotypical changes. [27]

Roat et al. showed that children with DS have lower percentages of sjTREC positive cells in PBMC compared to controls. [17,28] They suggested that this was the result of thymic

impairment. Although this conclusion could explain their observation, sjTREC dynamics and T-cell homeostasis are also influenced by cellular lifespan and division. [29] In the current study, we addressed naive T-cell dynamics in children with DS now incorporating thymic output, T-cell proliferation and T-cell loss by antigen driven differentiation or apoptosis. From our combined results we conclude that decreased thymic output is responsible for the low naive T-cell numbers in children with DS.

METHODS

Study population

Whole blood samples were obtained by venapuncture from healthy children with DS and controls. Forty-seven children with DS who attended the outpatient clinic of the Wilhelmina Children's Hospital Utrecht, The Netherlands were included in six age groups from 0.1-12 years. Since the largest developmental changes in the immune system are described in approximately the first two years of life, three times more children were included in the age group of 0-2 years (n=16) compared to the other age groups, in which 5 children per 2-year stratum were included. [30,31] Children with DS with a history of surgery in the previous 2 months were excluded. An equal number of age-matched, otherwise healthy children, who were admitted to the hospital for elective urologic, plastic, ophthalmologic or general surgery, were included as controls. To minimize interference on immunologic parameters blood was drawn prior to or directly after anesthesia was given. None of the children showed signs of acute infection at the time that blood was drawn. Five children with DS and eight control children used prophylactic antibiotics at the time of sampling. Four to ten ml blood was drawn from each individual. In four children with DS and three controls the absolute lymphocyte count could not be determined. PBMC could not be isolated at all in three children of both groups. FACS staining could not be performed in four children with DS and six healthy controls and only partially in some of the 40 respectively 38 children left. For all cases parental written informed consent was obtained. The research protocol was approved by the medical ethics committee of the University Medical Centre Utrecht.

Cell preparation and cultures

PBMC were isolated from heparinized blood samples and plasma was isolated from EDTA-anticoagulated blood samples. PBMC were obtained by Ficoll-Paque density gradient centrifugation and stored in liquid nitrogen until further processing. $CD4^+$ and $CD8^+$ T-cells were purified from thawed PBMC by magnetic-bead separation using the MiniMACS multisort kit according to manufacturer's instructions (Miltenyi Biotec Inc). To measure sjTREC content within naive ($CD27^+CD45RO^-$) and memory ($CD27^+CD45RO^+$)

$CD4^+$ and $CD8^+$ T-cells, these subsets were isolated by cell sorting on a FACSAria (Becton Dickinson Biosciences (BD). PBMC were cultured with or without IL-7 at a final concentration of 10ng/ml IL-7 (Sigma) in RPMI 10% FCS culture medium for seven days. For the apoptosis assay, cryopreserved PBMCs were thawed and cells were either stained directly with the apoptotic marker Annexin V (BD) or after overnight culture in medium.

Flow cytometry

Thawed cryopreserved PBMCs were used for characterization of the T-cell compartment. PBMC were stained with monoclonal antibodies to CD3-Pacific Blue, CD4-Pacific Blue, CD4-PerCP-Cy5.5, CD4-APC-Cy7, CD8-PerCP-Cy5.5, CD8-APC-Cy7, CD8-Amcyan, CD25-PE, CD27-APC, CD27-APC-Cy7, CD31-PE, CD38-PerCP-Cy5.5, CD45RO-PE-Cy7, CD127-PE, HLA-DR-FITC, HLA-DR-PE (BD). Intracellular staining was performed to measure expression of Ki67 (Dako). In short, PBMCs were stained, fixed and permeabilized with cytofix/cytoperm (BD) and washed twice with Permeabilization wash buffer. Next cells were stained with antibody directed against Ki-67, washed twice with Permeabilization buffer and resuspended in FACS-buffer. Affinity-purified rabbit IgG anti-mouse PTK7 was kindly provided by X. Lu from the Department of Cell biology, University of Virginia, Charlottesville, USA. For surface PTK7 staining alexa fluor 488-conjugated goat anti-rabbit IgG, was used as a secondary antibody. As a control for aspecific binding cells were stained with an identical mixture without the anti-mouse PTK7 antibody. Annexin V staining was used according to manufacturer's protocol to determine the fraction of apoptotic cells. In short, after surface staining of T-cell subsets, cells were washed, incubated with Annexin V-antibodies for 15 minutes and washed again. Cells were resuspended in Annexin V buffer and analyzed. Forward/sideward scatter was used to exclude dead cells. Cellular fluorescence was measured using a LSRII flowcytometer (BD) and analyzed with FACS Diva software (BD). For each sample a minimum of 100.000 events within the lymphocyte gate were collected. For the rare subsets within $CD4^+$ and $CD8^+$ T-cells, we have used a minimum of 50 positive events to validate the percentage shown.

Population definitions

$CD8^+$ $CD3^+$ T-cells were subdivided into naive ($CD27^+CD45RO^-$) (N), memory ($CD27^+CD45RO^+$) (M), memory effector ($CD27^-CD45RO^+$) (EM) and effector ($CD27^-CD45RO^-$) (E) subsets. [32] In analogy, we have used the same definition of naive and antigen experienced subsets for $CD4^+$T-cells. In addition, in a limited number of patients expression of CD25, CD31, CD38, HLA-DR, CD127, Ki67, and PTK7 within naive and memory subsets was determined. Examples of the gating strategy used are shown in supplemental figure 1. Absolute lymphocyte count was determined using patented Multi Angle Polarized Scatter Separation (MAPSS™) *plus* 3-color Fluorescent on a CELL-DYN Sapphire™ (Abbott Diagnostics) and were used to calculate absolute numbers

of the indicated lymphocyte subsets by multiplying the percentage of the subset as obtained by flow cytometry with the absolute lymphocyte number.

sjTREC analysis

sjTREC numbers were determined by real-time PCR on genomic DNA of $CD4^+$ and $CD8^+$ T-cells as described previously. [29,33] DNA was purified using the NucleoSpin Blood QuickPure according to manufacturer's instructions (Machery-Nagel). sjTREC content per $CD4^+$ or $CD8^+$ T-cell was calculated by dividing the sjTREC content per µg DNA by 150.000 (assuming that 1µg DNA corresponds with 150.000 T-cells). Total numbers of sjTRECs were calculated as the sjTREC content per $CD4^+$ or $CD8^+$ T-cell multiplied by the absolute $CD4^+$ or $CD8^+$ T-cell count per ml.

IL-7 enzyme-linked immunosorbent assay

Plasma samples were frozen 3-12 hours after they were obtained. A short half-life of IL-7 might influence the results of our tests using samples with a wide time-frame before freezing. To exclude time as a possible dependent factor in the results of our test, we examined the influence of time until freezing of the sample on IL-7 plasma levels in three healthy donors. No significant differences were found in IL-7 plasma levels of samples frozen between 3-12 hours after sampling (data not shown). None of the samples had been thawed previously. After thawing, samples were analyzed using an enzyme-linked immunosorbent assay (Quantikine HS, R&D systems) according to the manufacturer's recommendations. All samples were run in duplicate. A standard curve was prepared by serial dilutions.

Statistical analysis

Differences in results between children with DS and healthy controls were compared using Chi Square test, Student's t-test or non-parametric tests in case of unequal distribution between the groups. Values are expressed as the means ± SEM. In all analysis regression analysis including sex and age was performed to evaluate whether differences existed between children with DS and healthy controls. All statistical analyses were performed using the software program SPSS for Windows (version 12.0.2; SPSS Inc., Chicago, IL). A P value of ≤ 0.05 was considered significant. A sensitivity analysis was performed to determine if a history of corrective surgery for congenital heart disease in children with DS influenced the outcome of the results of this study.

RESULTS

Forty-seven children with DS, age 0-12 years were included in the study. An equal number of healthy age-matched children admitted to the hospital for elective surgery were included as controls. Children were divided in eight age strata of two years with 5-6 children each. We used narrower strata in the first two years of life, because the largest immunological changes are found during those years. Because of restrictions in blood volume that could be drawn and concomitant yield of PBMC, some experiments could not be performed in all 47 children (for details see methods). In our study group of children with DS we had 20 children with DS with congenital heart disease in some degree. Six of them had no corrective surgery. In four children only IL-7 ELISA has been performed. Ten out of fourteen children, who had corrective heart surgery, had an age above 6.5 years when blood was drawn.

Decreased absolute numbers of naive CD4$^+$ and CD8$^+$ T-cells

We confirmed that children with DS have lower absolute numbers of naive CD4$^+$ and CD8$^+$ T-cells compared to healthy controls (0.91 versus 1.84 x 10^9/l naive CD4$^+$, p<0.001 and 0.41 versus 0.83 x 10^9/l naive CD8$^+$, p<0.001) (figure 1a, b). [8-13] This effect was seen at all ages, although differences between groups became smaller with increasing age. Absolute numbers of naive T-cells were negatively correlated with age in both groups. Normal absolute numbers of memory and effector subsets of CD4$^+$ and CD8$^+$ T-cells were found in children with DS compared to controls (supplemental figure 2a, b).

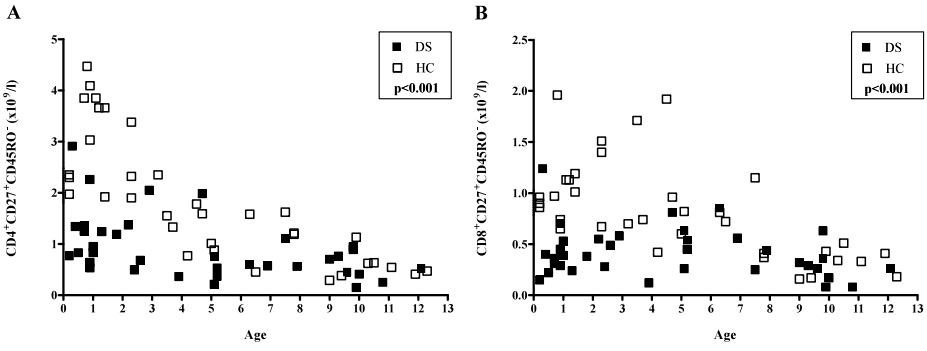

Figure 1. Children with DS have decreased absolute naive T-cell counts.
Age distribution of absolute numbers of naive CD4$^+$ (A) and naive CD8$^+$ (B) T-cell subsets in children with DS (n=36) compared to HC (n=35) . Regression analysis showed independent effects of DS and age.

Decreased sjTRECs and RTE numbers in children with DS

Several studies have shown thymic abnormalities in children with DS. To assess the cause of low absolute numbers of naive T-cells in children with DS, we firstly measured total sjTREC numbers per ml of blood. Both for $CD4^+$ and $CD8^+$ T-cells we found total sjTREC numbers to be more than two fold decreased in children with DS compared to controls (mean 0.23 vs. 0.50 x 10^9/l, p=0.003 and 0.07 vs. 0.13 x 10^9/l, p=0.001, respectively) (figure 2a, b). Regression analysis of total sjTREC numbers in $CD4^+$ and $CD8^+$ T-cells showed independent effects of DS and age.

Next, we measured the absolute number and percentage of $CD4^+$ RTEs as determined by the expression of PTK7 on naive $CD4^+$ T-cells. In children with DS the absolute number per ml blood and the percentage of PTK7 positive cells within naive $CD4^+$ T-cells was decreased (0.10 vs. 0.32 x 10^9/l, p<0.001 and 10.2 vs. 18.6%, p=0.008 respectively, figure 2c, d). Regression analysis of both absolute numbers and percentages of PTK7 positive cells within naïve $CD4^+$ T-cells showed independent effects of DS and age.

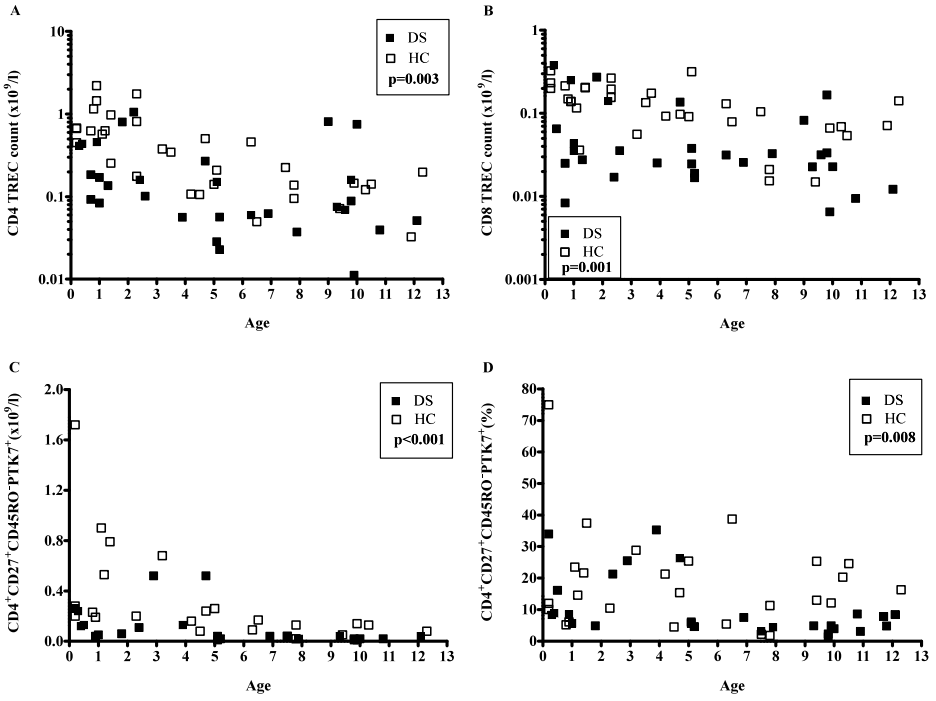

Figure 2. Children with DS have lower TREC numbers and RTE numbers and frequency compared to HC.
Age distribution of $CD4^+$ (A) and $CD8^+$ (B) TREC counts per ml blood, and absolute numbers (C) and percentages (D) of $PTK7^+$ RTE within naive $CD4^+$ T-cells in children with DS (n=30 respectively n=30, n=24 and n=27) compared to HC (n=32 respectively n=32, n=23 and n=26). Regression analysis showed independent effects of DS and age.

No increased loss of peripheral naive T-cells in DS

To determine whether reduced thymic output or increased loss causes the observed reduction in sjTREC numbers and RTE, we measured the level of peripheral naive T-cell loss by flow cytometry. We analyzed the percentage of naive $CD4^+$ and $CD8^+$ T-cells expressing the apoptosis marker Annexin V. Annexin expression was analyzed ex vivo and after 16 hrs of culture in the presence or absence of polyclonal stimulation with anti CD3 mAb. Under all circumstances there was no difference between the fraction of apoptotic naive $CD4^+$ and $CD8^+$ T-cells between children with DS and healthy controls (figure 3a, b).

Next we analyzed the level of antigen-induced activation. We reasoned that, as seen in HIV infection, increased antigen-induced-activation could lead to naive T-cell loss. The children with DS in this study did not show any clinical signs of acute or chronic infection. We used expression of CD25 in naive T-cells as a measure of recent antigenic activation of $CD8^+$ T- cells. [34] No differences were found in the absolute number of recent ($CD25^+CD45R0^-CD27^+$) $CD8^+$ T-cells (figure 3c). Analysis of T-cell activation using the activation markers $CD38^+$ and HLA-DR^+ gave also no indications for increased T-cell activation as the absolute numbers of $CD38^+$HLA-DR^+ $CD4^+$ and $CD8^+$ T-cells were similar in DS children compared to healthy controls (supplemental figure 3a, b). Since we found no indications of increased peripheral naive $CD4^+$ or $CD8^+$ T-cell loss we concluded that in children with DS the reduction in sjTREC numbers and RTE resulted from decreased thymic output.

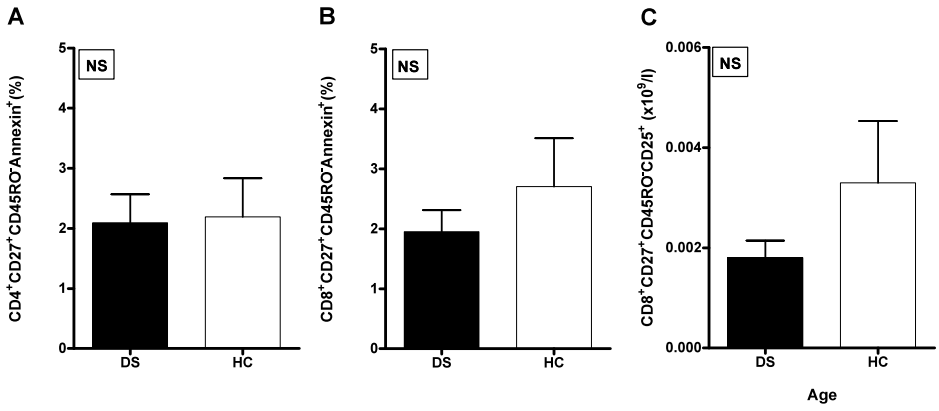

Figure 3. Children with DS have normal peripheral loss of naive $CD4^+$ and $CD8^+$ T-cells.
The fraction of Annexin V^+ naive $CD4^+$ (A) and $CD8^+$ (B) T-cells was used as a marker for apoptosis by flow cytometry in children with DS (n=24 respectively n=23) and HC (n=28 respectively n=26). The absolute numbers of $CD25^+$ naive $CD8^+$ (C) T-cells was determined as a measure for antigen-induced activation by flow cytometry in children with DS (n=28) and HC (n=21).

No indication for reduced peripheral generation of naive T-cells

Besides thymic output, peripheral proliferation contributes largely to the establishment of the naive T-cell pool. [35-37] We assessed possible disturbances in peripheral proliferation using the proliferation marker Ki67. Normal absolute numbers (supplemental figure 4a, b) and percentages of naive $CD4^+$ T-cells and naive $CD8^+$ T-cells expressing Ki67 were found in children with DS compared to controls (figure 4 a and b).

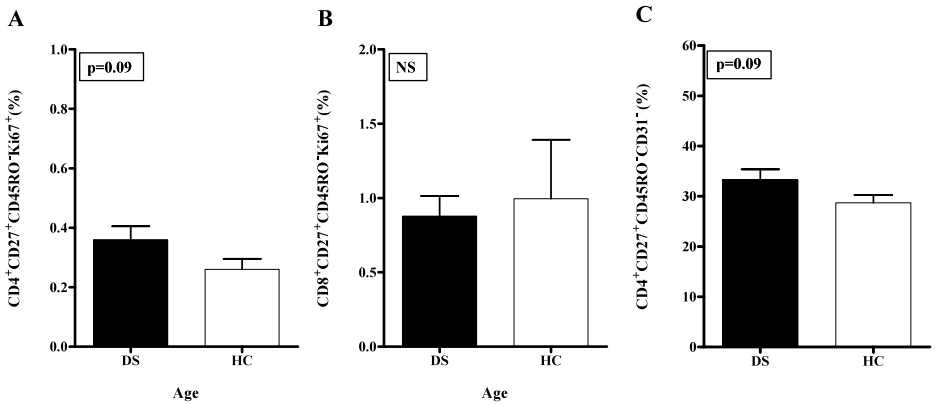

Figure 4. Children with DS show normal peripheral generation of naive $CD4^+$ and $CD8^+$ T-cells.
Flow cytometric analysis of relative numbers of $Ki67^+$ naive $CD4^+$ (A) and $CD8^+$ (B) T-cells in children with DS (n=37) and HC (n=37).
Flow cytometric analysis of relative numbers of $CD31^-$ naive $CD4^+$ (C) T-cells in children with DS (n=40) and HC (n=37).

For naive $CD4^+$ T-cells, CD31 is a thymic proximity marker, and loss of CD31 expression has been suggested to reflect self antigen driven proliferation. [24,25 27] In line with the Ki-67 data, analysis of the fraction of CD31 negative expressing cells among naive $CD4^+$ T-cells was similar compared to healthy controls (33.3 vs. 28.7%, p=0.09) (figure 4c) Thus both analysis showed no evidence for reduced peripheral proliferation as a cause for the reduced naive T-cell numbers in DS.

No indication for defective IL-7 dependent peripheral proliferation or survival

IL-7 is a cytokine important for T-cell survival and proliferation, especially of naive T-cells. [23] Its production is regulated by an IL-7R mediated feedback loop, which together with the utilisation by T-cells determines the serum and tissue levels of the cytokine. In human lymphopenic settings IL-7 plasma levels are increased and are thought to increase low affinity TCR induced proliferation and enhance survival of naive T-cells. [38-40] We investigated whether IL-7 influenced peripheral naive T-cell numbers in DS. In children with DS significantly higher IL-7 plasma levels were found, which was not effected by age (7.5 vs. 5.8 pg/ml, p=0.03) (figure 5a). However, no correlation between IL-7 plasma level and number of total (supplemental figure 5a, b) and naive $CD4^+$ or $CD8^+$ T-cells was

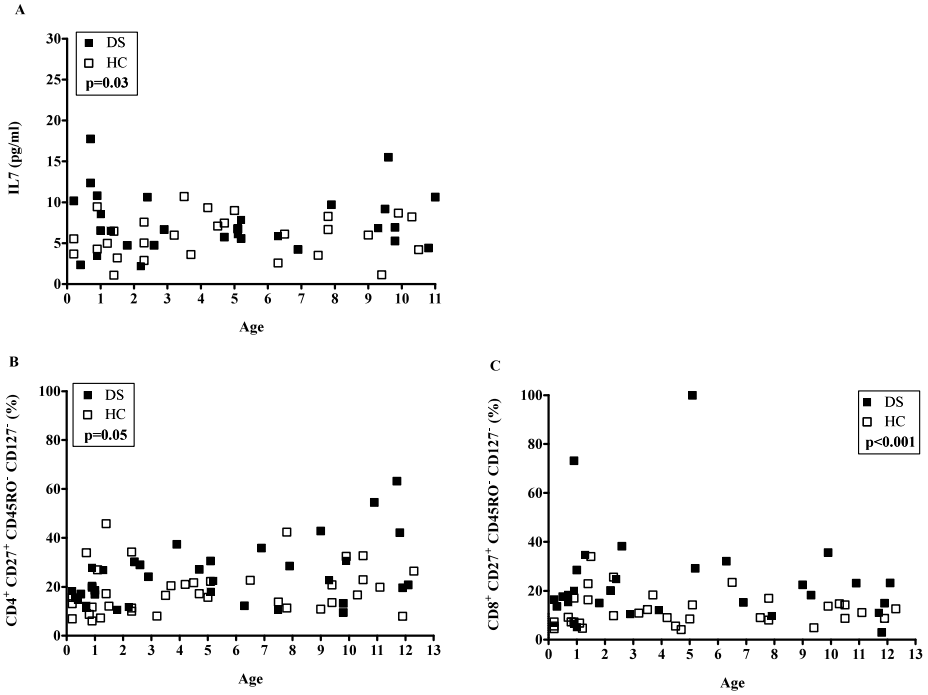

Figure 5. Children with DS have normal IL-7 dependent peripheral proliferation and survival.
Plasma levels of IL-7 (A) measured by ELISA in children with DS (n=29) compared to HC (n=35) at different ages. Regression analysis showed no effect of age. Flow cytometric analysis of relative numbers of $CD127^-$ naive $CD4^+$ (B) and $CD8^+$ (C) T-cells within the respective naive T-cell pools in children with DS (n=36 respectively n=30) and HC (n=37 respectively n=34). Regression analysis showed independent effects of age and DS in the CD4 subset and no effect of age in the CD8 subset

found (supplemental figure 5c, d). In vitro stimulation with IL-7 showed a normal capacity to respond to IL-7 in children with DS, as down modulation of CD127, the limiting component of the IL-7 receptor was similar (data not shown). Ex vivo analysis of CD127 expression on naive T-cells showed small increases in relative fraction of $CD127^-$ within naive $CD4^+$ (figure 5b) and $CD8^+$ (figure 5c) subsets (24.6 vs. 18.9%, p=0.05 respectively 23.6 vs. 12.0%, p<0.001), suggestive of increased per cell IL-7 utilization and increased survival or proliferation. Regression analysis showed independent effects of age and DS in the CD4 subset and no effect of age in the CD8 subset. Thus despite increased IL-7 plasma levels we found no indications for a role of IL-7 in decreasing peripheral proliferation in children with DS.

Cumulative effects over time: sjTREC content analysis

To corroborate our conclusion that reduction in peripheral proliferation and increase of naïve T-cell loss are not the cause of reduced naive T-cell numbers in children with DS, we measured sjTREC content of the $CD4^+$ and $CD8^+$ T-cell compartment (supplemental

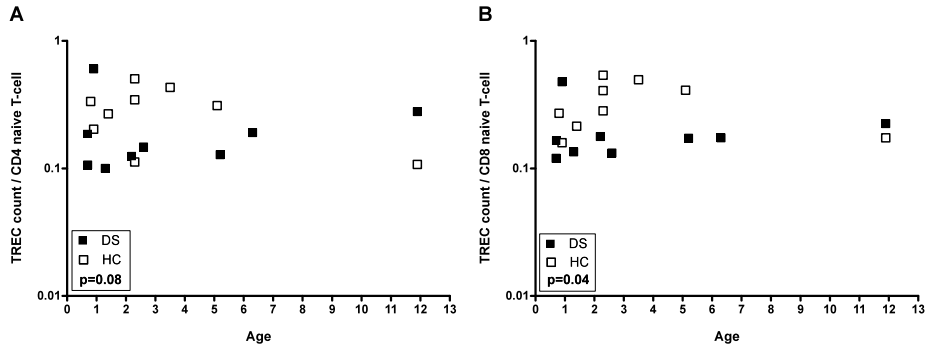

Figure 6. Children with DS have normal or even decreased TREC content compared to HC.
To determine whether lower TREC counts in $CD4^+$ and $CD8^+$ T-cells reflect lower fraction of naive T-cells within children with DS, TREC contents of purified naive $CD4^+$ (C) and $CD8^+$ (D) T-cells were measured in children with DS (n=9) and HC (n=9). Regression analysis showed independent effects of age and DS.

figure 6). In contrast to sjTREC numbers per ml blood, which reflect the balance between thymic production and T cell loss, sjTREC content per $CD4^+$ or $CD8^+$ T-cell reflects the balance between thymic output, T cell proliferation and loss and small changes in proliferation or survival rates will accumulate over time in larger differences in the sjTREC content. When peripheral proliferation or survival per cell is decreased, the sjTREC content per T-cell will become relatively higher. In agreement with our hypothesis, no significant changes were found in the sjTREC content of sorted naive $CD4^+$ T-cells in children with DS (figure 6a). For sorted naive $CD8^+$ T-cells (figure 6b) decreased sjTREC content was found in children with DS compared to healthy controls. Regression analysis showed independent effects of age and DS. The decreased sjTREC content in sorted naïve $CD8^+$ T-cells is also in line with our hypothesis and even suggests increased proliferation or decreased loss in children with DS.

DISCUSSION

Histopathologic studies of thymic tissue in DS have shown increased involution of the thymus and altered patterns of maturation of thymocytes. [19,21,22] In addition, several studies have described low numbers of T-cells, decreased cytokine production upon antigenic stimulation and diminished antibody-dependent T-cell-mediated cytotoxicity in DS. [10,13,41] It was suggested that thymic insufficiency causes peripheral T-cell dysfunction in DS and that decreased thymic output is a causative factor for the low numbers of naive T-cells in these children. sjTREC analysis is the most applicable tool to determine thymic output in humans, but the results should be interpreted with care. [29] Reduced

sjTREC contents might be indicative of reduced thymic output, but are also influenced by peripheral mechanism of T-cell maintenance. Since sjTRECs are circles of DNA spliced off during T-cell receptor rearrangement that do not replicate during mitosis, they are diluted upon cell division. In addition, longevity of naive T-cells influences sjTREC content of cells by decreasing sjTREC content when naive T-cells tend to live longer. For this reason absolute numbers of sjTRECs per millilitre blood are used to provide information on thymic output, whereas the average sjTREC content per cell is used for determination of the replicative history. The combined results of our in detail study of T-cell dynamics now put forward that in children with DS decreased thymic output is solely responsible for the decreased naive T-cell numbers, and that peripheral mechanisms of naive T-cell maintenance are fully functional in children with DS. In fact, based on the reduced sjTREC content we conclude that peripheral mechanisms counteract the reduced thymic output. Future studies on thymopoiesis including analysis of thymic tissue, in particular of T-cell subsets, their reactivity to IL-7, intrathymic precursor T-cell proliferation and the implication of growth hormones in thymopoiesis might clarify the cause of decreased thymic output in children with DS.

We are the first to report on RTEs identified by PTK7 in a large paediatric population after its description by Haines et al. [26] Within this healthy pediatric cohort percentages of RTE were similar to percentages described by Haines. Regression analysis showed an age dependent decline in the absolute numbers but not frequency of PTK7 positive cells within naive $CD4^+$ T-cells in the blood between the age of 0 and 13 years. This age independent frequency of RTE implies that no RTE specific turnover changes take place with age and therefore that RTE and resident naive T-cells are mostly regulated by the same mechanisms.

In previous DS literature, accelerated thymic involution and clinical findings such as occurrence of Alzheimer's disease at the young age of 40 to 50 years, have been ascribed to early senescence. Ageing of the immune system is difficult to define, but decline in naive T-cell numbers, oligoclonal expansion of memory T-cells starting around the sixth decade of life and loss of TCR diversity are important aspects. [42] Although the decreased numbers of naive T-cells support the concept of accelerated ageing, other results are in apparent contrast. First, in accelerated ageing one would expect that absolute naive T-cell numbers and total sjTREC counts are lower and keep declining over age at a higher rate than in controls. In children with DS, a higher rate of decline was only found in the first two to four years. Secondly, with ageing, naive and central memory $CD8^+$ T-cells are lost while $CD8^+$ effector T-cells increase. [43,44] In children with DS, over age no significant changes in absolute or relative numbers of $CD8^+$ memory and effector T-cells were shown. A third aspect of ageing is loss of TCR diversity caused by oligoclonal expansion, mostly within memory $CD8^+$ T-cells. When we used Vβ spectratyping to compare oligoclonality of PBMC of children with DS and healthy controls, a larger number of TCR Vβ families

that were oligoclonal were seen in children with DS (unpublished results). Since TCR diversity was not measured within $CD4^+$ and $CD8^+$ T-cell subsets, it is unclear whether this loss of TCR diversity found in children with DS reflects a relative increase of memory T-cells or clonal expansion associated with homeostatic maintenance of the naive T-cell compartment or a combination of both. If loss of TCR diversity in children with DS is not explained by early senescence, it might still play a role in the higher susceptibility to infections in children with DS. Future studies are needed to explore TCR diversity and functional loss of the immune system at different ages to further clarify the concept of ageing in patients with DS. Future longitudinal studies that correlate these immunologic parameters to clinical data could give better insight in the role of decreased naïve T-cells in the morbidity found in children with DS.

The natural variance in values of the dynamic parameters, even in a healthy population, can be quite substantial (about 4 fold, up to 10 fold for TREC content). Next to genetic disposition, factors like age and infection status are known effectors. Recent or chronic infections were exclusion criteria for our study, to prevent the effect of infection status. Despite our relative small group sizes and the large variance we were able to find significant age effects and differences between DS and healthy controls. More important, for all results that were significantly different between DS and healthy controls, regression analysis showed an effect of DS that was independent of age. Based on the difference between DS and healthy controls, we concluded that thymic output and not disturbed peripheral generation is responsible for the decreased number of naïve T-cells in children with DS. In line with this conclusion, none of our analysis suggested intrinsic defects in cell death or proliferation or increased T-cell activation that would explain the reduced T- cell numbers in DS. In contrast, in DS trends towards decreased numbers of $CD25^+$ and percentages of Annexin V^+ naïve T-cells and increased frequencies of $Ki67^+$ and $CD31^-$ naïve T-cells were found, which would suggest compensatory peripheral mechanisms for the reduced thymic output. In addition to age and infection status, corrective surgery for congenital heart disease with the risk of thymectomy might have influenced the results of our study. A sensitivity analysis comparing children with DS without corrective heart surgery and healthy controls confirmed the conclusions from all analyses.

In conclusion, this is the first study that formally shows decreased thymic output as the cause of the diminished naive T-cell pool in children with DS, confirming the hypothesis of thymic insufficiency. Evidence of an intrinsic naive T-cell defect contributing to a diminished naive T-cell pool in these children could not be provided. Our data suggested that IL-7-driven peripheral mechanisms might counteract the reduced thymic output. Future functional studies are needed to determine if increased peripheral proliferation and reduced TCR diversity of naive T-cells have a role in the morbidity described in DS.

Acknowledgments

We would like to thank P. van der Weide from the Department of Pathology, University Medical Centre Utrecht, The Netherlands for her excellent technical assistance, F. Miedema from the Department of Immunology, University Medical Centre Utrecht, The Netherlands for his useful comments on the research and manuscript and X. Lu from the Department of Cell biology, University of Virginia, Charlottesville, USA for kindly providing the affinity-purified rabbit IgG anti-mouse PTK7.

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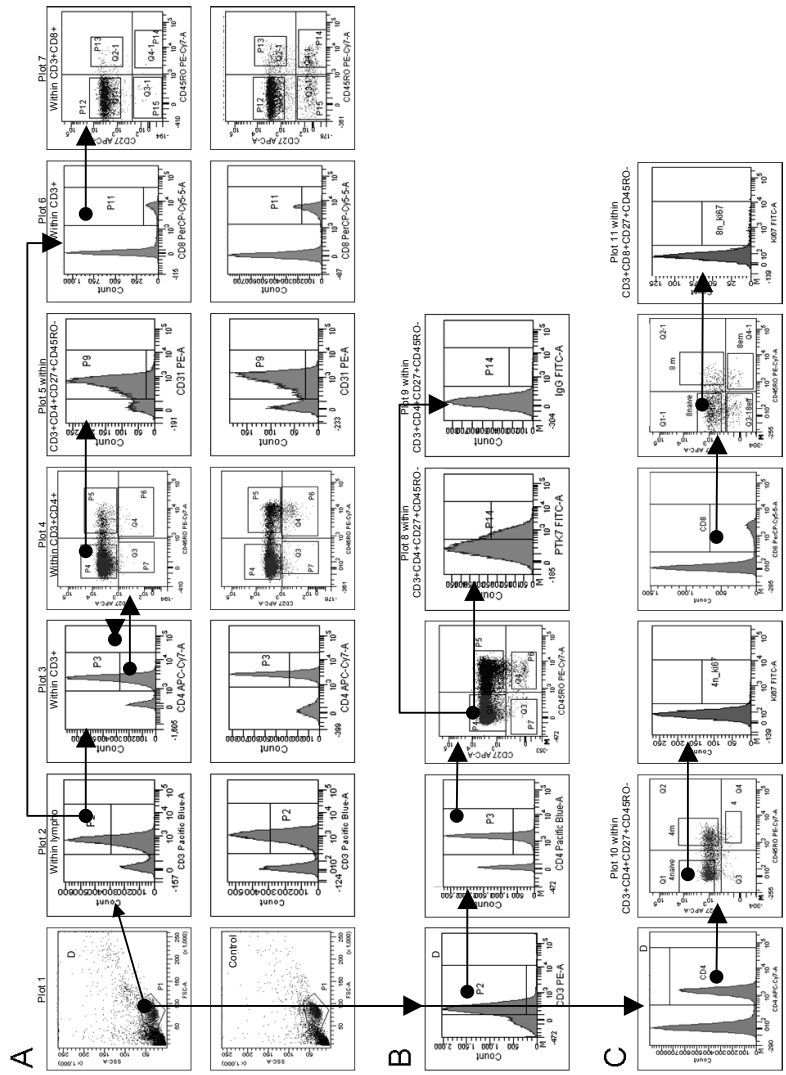
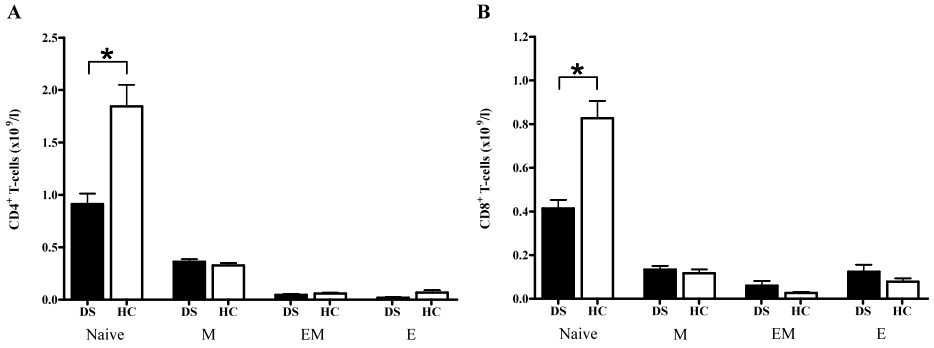
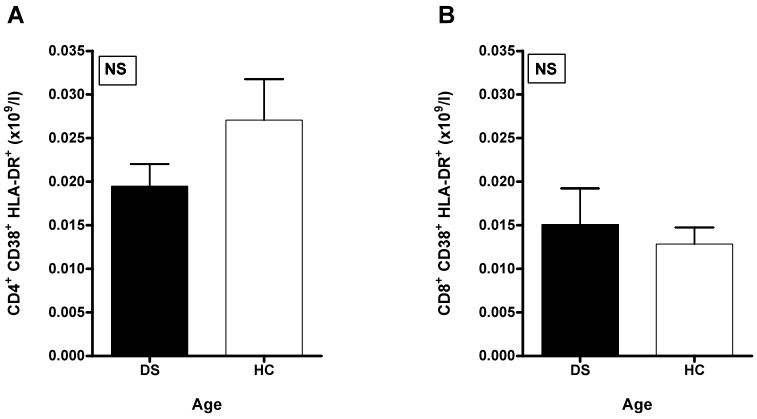
SUPPLEMENTAL FIGURES



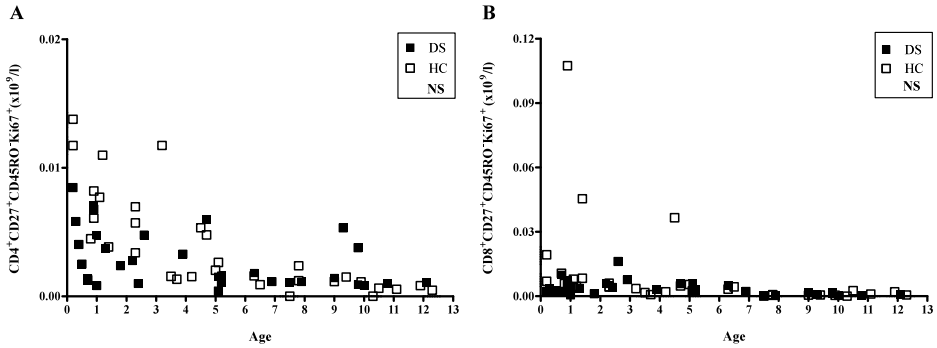
Supplemental figure 1. Characterization of T-cell subsets. (A) Representative examples of the gating strategy used for the measurements of the different T-cell subsets and the marker expression within these subsets as described in the Materials and Methods section for a child with DS (upper plots) and a healthy control (lower plots). Lymphocytes were defined based on their forward and side scatter (P1, plot 1) and $CD3^+$ T-cells (P2, plot 2) were gated within this lymphocyte gate. $CD4^+$ (P3, plot 3) and $CD8^+$ (P11, plot 6) T-cells were defined as $CD4^+$ and $CD8^+$ cells within the $CD3^+$ lymphocyte gate. Within the $CD4^+$ (P3) and $CD8^+$T-cells (P11) naive $CD27^+CD45R0^-$ (P4 respectively P12, in plot 4 and 7), memory $CD27^+CD45R0^+$ (P5 respectively P13, in plot 4 and 7), memory effector $CD27^-CD45R0^+$ (P6 respectively P14, in plot 4 and 7) and effector $CD27^-CD45R0^-$ (P7 respectively P15, in plot 4 and 7) subsets are shown. CD31 expression within naive $CD27^+CD45R0^-CD4^+$ T-cells (P4) is shown in plot 5. (B) A representative example of the gating strategy for the determination of the fraction of PTK7 expressing cells within $CD27^+CD45R0^-CD4^+$ T-cells (P4) is shown (plot 8) with the isotype control (plot 9) in a child with DS. (C) A representative example of the gating strategy for the determination of the percentage of Ki67 expressing cells within naïve $CD27^+CD45R0^-CD4^+$ (plot 10) and $CD8^+$ (plot 11) T-cell subsets is shown in a child with DS.



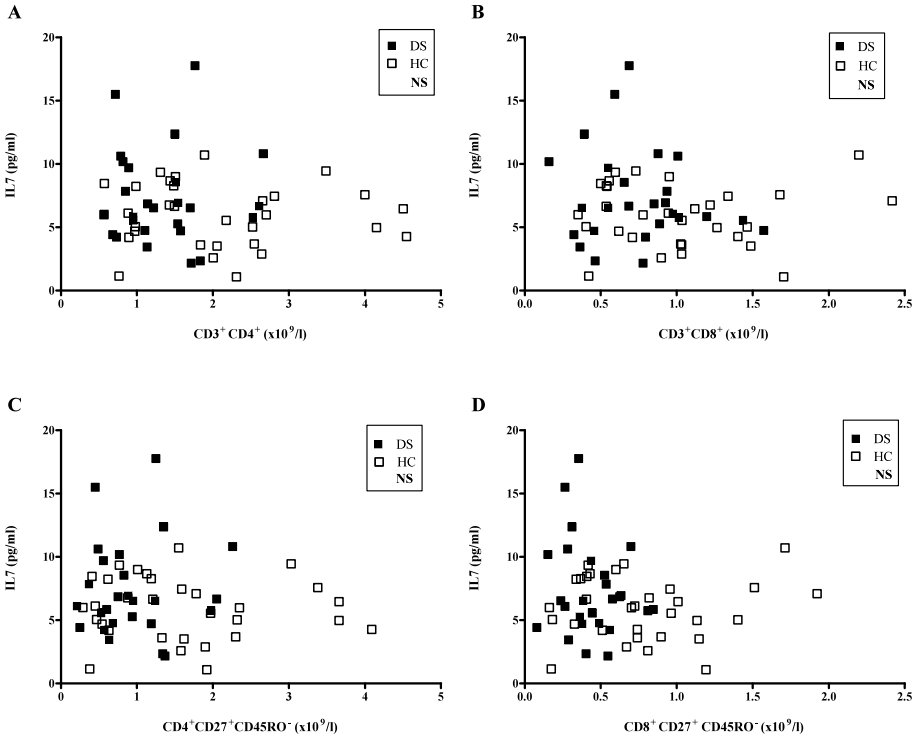
Supplemental figure 2. Children with DS have normal numbers of memory and effector T-cells.
Pooled data of absolute numbers of $CD4^+$ (A) and $CD8^+$ (B) T-cell subsets in children with DS (n=36)) compared to HC (n=35) (mean ± SEM). *Significant difference ($P < .001$)
N, naïve; M, memory; EM, memory effector; E, effector



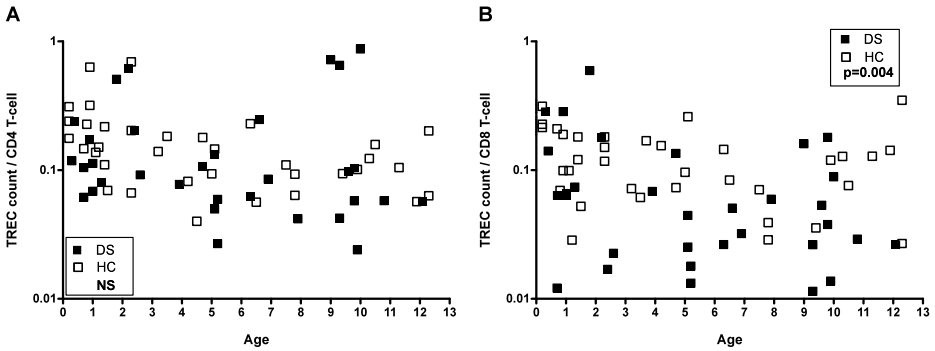
Supplemental figure 3. Children with DS have normal activation of naive $CD4^+$ and $CD8^+$ T-cells.
The absolute number of $CD38^+$ $HLA\text{-}DR^+$ $CD4^+$ (A) and $CD8^+$ (B) T-cells was determined by flow cytometry in children with DS (n=33) and HC (n=35).



Supplemental figure 4. Children with DS have normal peripheral generation of naive $CD4^+$ and $CD8^+$ T-cell subsets.
Flow cytometric analysis of absolute numbers of $Ki67^+$ naive $CD4^+$ (A) and $CD8^+$ (B) T-cells in children with DS (n=33) and HC (n=34) at different ages.



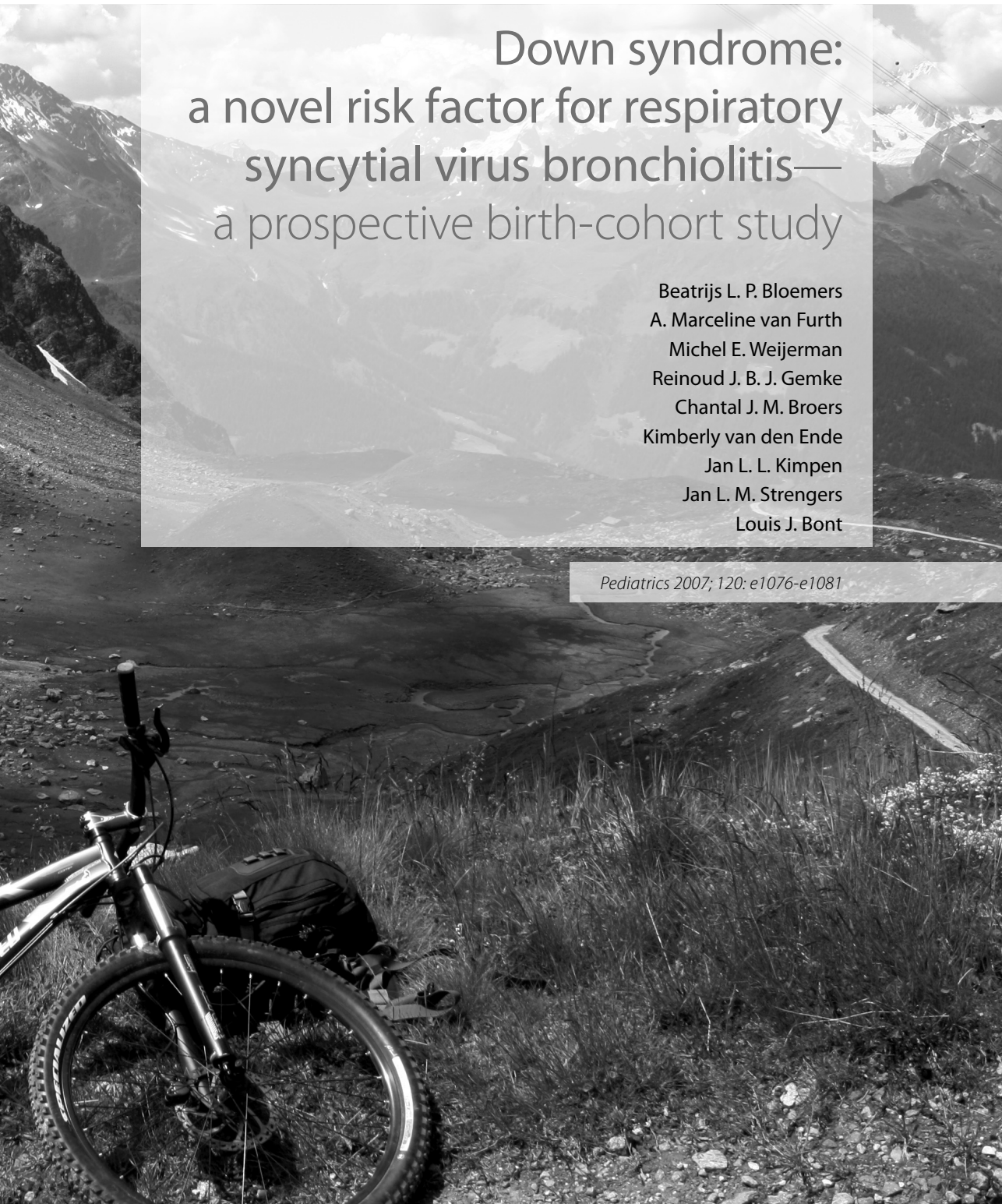
Supplemental figure 5. Children with DS show no correlation of IL-7 plasma levels and absolute T-cell numbers.
Correlation between IL-7 plasma levels and absolute numbers of total and naive $CD4^+$ (A, C) and $CD8^+$ (B, D) T-cells in children with DS (n= 26) compared to HC (n=31).



Supplemental figure 6. Children with DS have normal or even decreased TREC content compared to HC.
Age distribution of TRECs per $CD4^+$ (A) and $CD8^+$ (B) T-cell in children with DS (n=32) compared to HC (n=35) at different ages.


La Chaux (2260m)
– Col des Gentianes (2950m)

Chapter 5



Down syndrome: a novel risk factor for respiratory syncytial virus bronchiolitis—a prospective birth-cohort study

Beatrijs L. P. Bloemers
A. Marceline van Furth
Michel E. Weijerman
Reinoud J. B. J. Gemke
Chantal J. M. Broers
Kimberly van den Ende
Jan L. L. Kimpen
Jan L. M. Strengers
Louis J. Bont

Pediatrics 2007; 120: e1076-e1081

ABSTRACT

Objectives

Respiratory syncytial virus is the single-most important cause of lower respiratory tract infections in children. Preterm birth and congenital heart disease are known risk factors for severe respiratory syncytial virus infections. Although Down syndrome is associated with a high risk of respiratory tract infections, little is known about the incidence of respiratory syncytial virus infections in this group. The aim of our study was to determine the incidence of respiratory syncytial virus lower respiratory tract infection-associated hospitalization among children with Down syndrome.

Methods

We performed a retrospective observational study and a prospective nationwide birth-cohort study of children with Down syndrome. The retrospective cohort comprised 176 children with Down syndrome. A birth cohort of 219 children with Down syndrome was prospectively followed until 2 years of age. All 276 siblings of the birth cohort were used as controls.

Results

Of the 395 patients with Down syndrome, 180 (45.6%) had a known risk factor for severe respiratory syncytial virus infections; 39 (9.9%) of these were hospitalized for respiratory syncytial virus lower respiratory tract infections. Two control children (0.7%) versus 9 term children with Down syndrome without congenital heart disease (7.6%) were hospitalized for respiratory syncytial virus lower respiratory tract infections. The median duration of hospitalization was 10 days; mechanical ventilation was required for 5 children (12.8%).

Conclusions

This is the first study, to our knowledge, to demonstrate that Down syndrome is a novel independent risk factor for severe respiratory syncytial virus lower respiratory tract infections. These findings should prompt studies to investigate possible mechanisms that underlie severe respiratory syncytial virus lower respiratory tract infections in children with Down syndrome. The effect of respiratory syncytial virus prophylaxis in this specific population needs to be established.

INTRODUCTION

Respiratory syncytial virus (RSV) is the single-most important cause of lower respiratory tract infections (LRTIs) in infants and young children. [1] Virtually all children are infected with RSV before the age of 2 years, and 40% of RSV infections progress to LRTI. Approximately 0.5% to 2% of children require hospitalization (and 2.2% of these require mechanical ventilation), which makes RSV LRTI the most common cause of hospitalization among infants during winter. [2] Most hospitalized children are younger than 6 months. Premature birth, chronic lung disease, age <6 weeks, and congenital heart disease (CHD) are clinical risk factors for severe disease after RSV infection. [3-5] In addition, a decreased lung function at birth is hypothesized to have an important role in the pathogenesis of RSV LRTI. [6]

Down syndrome (DS) is the most common chromosomal abnormality among live-born infants and is characterized by a variety of dysmorphic features and congenital malformations. [7] It is associated with CHD, gastrointestinal disease, various immunological impairments, and concomitant respiratory pathology. [8-11] Because LRTI is the most common cause of acute hospitalization among children with DS, [12] we hypothesized that children with DS are at increased risk of severe RSV LRTI, and, hence, hospitalization. The primary aim of this study was to determine the incidence of RSV LRTI-associated hospitalization among children with DS with and without known risk factors.

METHODS

Study Design

A retrospective observational study of RSV LRTI-associated hospitalization in a cohort of children with DS was performed. Subsequently, the results of this study were validated in a prospective nationwide birth cohort of children with DS.

Study Population

Three groups were studied. First, a retrospective study was performed involving a cohort of children with DS who were being monitored by the Down Syndrome Study Group and who attended the outpatient clinic of the Pediatric Department of the VU University Medical Centre. The cohort comprised 206 children with DS, born between 1976 and 2005, with a median age of 6.5 years (range: 0.3-29.6 years). Patient charts were reviewed to determine the number of previous RSV LRTI-associated hospitalizations. The following information was obtained: gestational age, the presence of chronic lung disease of prematurity, congenital heart disease, cardiac surgery and post-operative hemodynamic status, and preexisting airway symptoms. The second study group consisted of a na-

tional birth cohort of 241 children with DS, born between 2003 and 2005, and followed until 2 years old. The cohort was prospectively collected by the Down Syndrome Study Group and TNO Quality of Life Leiden, under auspices of the Dutch Pediatric Surveillance Unit. This national registry has been established to facilitate research into the etiology, diagnostics, treatment, prognosis, and incidence of specific disorders and has coverage of 90 to 95%, depending on the disease. Children with DS are registered by their pediatrician. One of the investigators (Dr Bloemers) administered the children's primary caregiver a standardized questionnaire, by telephone, to acquire the same clinical information that was available for the first group. Although the primary aim of this study was to determine the absolute incidence of RSV LRTI-associated hospitalization in children with DS, a third, unmatched, control group was included. This control group was used to estimate the relative risk of RSV LRTI-associated hospitalization in children with DS. All 276 siblings born between 1976 and 2005 (median age: 5.6 years; range: 0-29.8 years) of the prospectively followed birth cohort were used as controls. Exclusion criteria for all 3 study groups were use of palivizumab (a humanized monoclonal antibody against RSV) and death not associated with RSV infection before the age of 2 years. Parental informed consent was obtained for the collection of all data.

RSV LRTI-Associated Hospitalization

RSV LRTI-associated hospitalization was defined as hospital admission for lower respiratory tract symptoms (deep or wet chest cough, wheezing, hoarseness, stridor, shortness of breath) and either a positive enzyme immunoassay for RSV, a positive direct immunofluorescence assay for RSV infection of epithelial cells in nasopharyngeal secretions or a positive viral culture for RSV. Age at time of diagnosis, total number of days in hospital, days in the ICU, days on mechanical ventilation, and days on supplemental oxygen were retrieved from hospital charts. RSV infection without hospitalization was not evaluated.

Risk Factors for RSV LRTI-Associated Hospitalization

Known clinical risk factors for RSV LRTI-associated hospitalization are hemodynamically significant CHD and prematurity (gestational age of <37 weeks) with or without chronic lung disease. Because DS is frequently associated with CHD, in the Netherlands all children with DS are routinely evaluated for CHD (and its hemodynamic relevance) by a pediatric cardiologist. For this study, the initial cardiologic evaluation of all children with CHD in the prospective group was reassessed by an independent cardiologist (Dr Strengers) who was unaware whether the children had developed RSV LRTI. Hemodynamically relevant CHD was defined as clinical signs and symptoms of left-right shunting or signs of volume overload because of left-right shunting on echocardiography. The results of the 2 evaluations were in almost perfect agreement. In the combined retrospective and prospective cohort, 63% of children with DS were found to have CHD,

and in 35.7% the CHD was hemodynamically significant. (Table 1) Of the children with hemodynamically relevant CHD, 18.7% had an atrio-ventricular septal defect (n=74), 6.1% a ventricular septal defect (n=24), 3.8% an atrial septum defect (ASD; n=15), 2.8% a patent ductus arteriosus (n=11), and 4.3% other hemodynamically relevant conditions (n=17). Hemodynamically insignificant CHD, defined as the absence of clinical or echocardiographic signs of increased pulmonary flow, included a patent foramen ovale or ASD type II (9.4%; n=37), small to moderate ventricular septal defect (4.8%; n=19) and/or ASD (7.3%; n=29), small patent ductus arteriosus (3.8%; n=15) and others (2.0%; n=8). In 64.9% of children with atrioventricular septal defect, the defect was (partially) corrected before 6 months of age. Prematurity, chronic lung disease, significant CHD and insignificant CHD were predefined risk factors.

Table 1. Baseline characteristics

Baseline Characteristic	Retrospective	%	Prospective	%	Total	%
Total patients, *N*	176	100	219	100	395	100
Male	108	61.4	123	56.2	231	58.5
< 37 wk, *N*	22	12.5	31	14.2	53	13.4
Gestational age, median, wk	34.5	-	35	-	35	-
Gestational age, range, wk	28-36	-	28-36	-	28-36	-
Severe comorbidity						
Total	13 *	7.4	9	4.1	22*	5.6
Hirschsprung disease	5	2.8	1	0.5	6	1.5
Duodenal atresia/web	6	3.4	8	3.7	14	3.5
Anal atresia	3	1.7	0	0.0	3	0.8
Chronic lung disease	0	0	1	0.5	1	0.3
CHD						
Total	103	58.5	146	66.7	249	63.0
Hemodynamically significant	57	32.4	84	38.4	141	35.7
Hemodynamically insignificant	46	26.1	62	28.3	108	27.3

* One child was diagnosed with both anal atresia and Hirschsprung disease

Statistical Analysis

The χ^2 test was used to compare the proportion of children with RSV LRTI-associated hospitalization between different populations and to calculate odds ratios. Differences in continuous nonparametric variables between populations were assessed using Mann-Whitney *U* test. Differences in duration of hospitalization for RSV LRTI among children with DS without comorbidity, CHD, and prematurity were analyzed with analysis of variance with Bonferroni correction. We were not able to perform a power analysis for this study, because no estimate of RSV LRTI-associated hospitalization was available. All statistical analyses were performed by using the software program SPSS for Windows 12.0.2 (SPSS Inc, Chicago, IL). A *P* value of .05 was considered the limit of significance.

RESULTS

Study Population

Clinical information was available for 176 of the 206 children in the retrospective cohort and for 219 of the 241 children in the prospective study cohort (Fig 1). Two children were lost to follow-up. A total of 395 children with DS were studied, and 231 (58.5%) were male. All children were followed until the age of 2 years. Baseline characteristics are outlined in Table 1. The third group, the control group, consisted of 276 unmatched siblings; 135 (48.9%) were male.

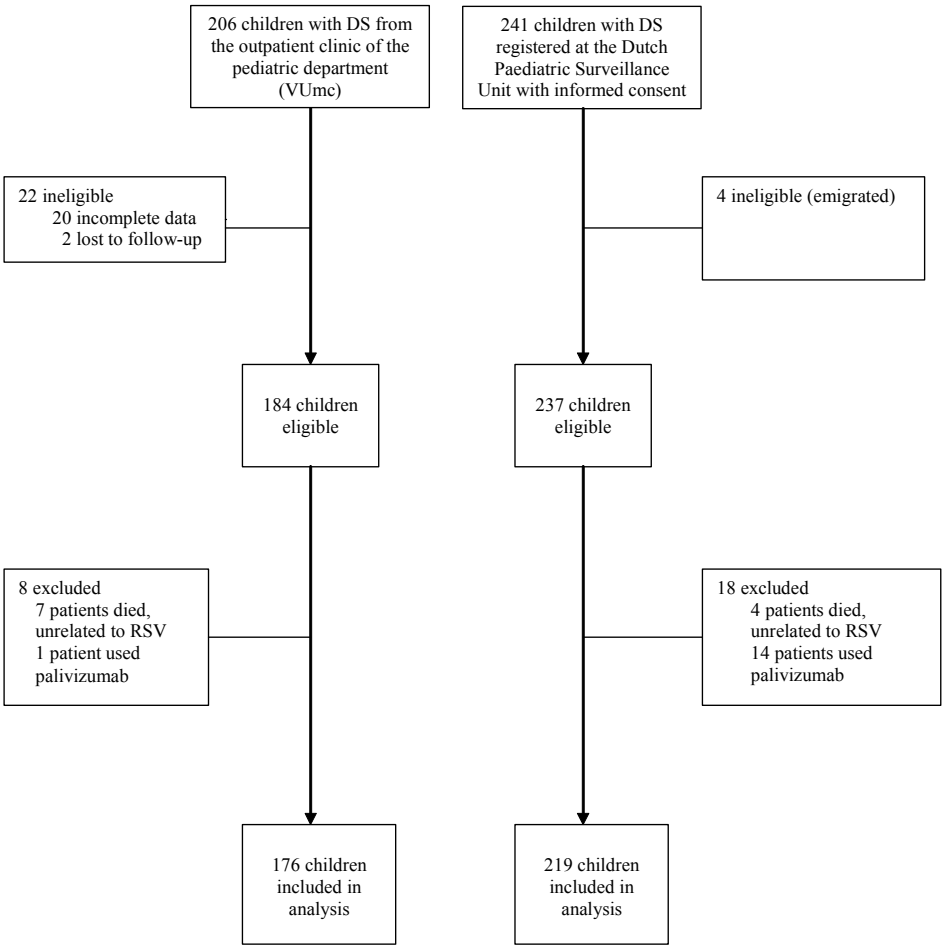


Figure 1. Selection of children in the analysis

DS indicates Down syndrome; VUmc, VU University medical centre; RSV, respiratory syncytial virus.

Risk Factors for RSV LRTI-Associated Hospitalization

In the retrospective cohort, 57 children (32.4%) had hemodynamically significant CHD, and 22 children were born prematurely, 6 of who had hemodynamically significant CHD. The latter children, therefore, had 2 risk factors for RSV LRTI-associated hospitalization. In the prospective cohort, 146 children (66.7%) had a congenital heart abnormality, detected during routine screening during the first weeks of life, and in 84 children (38.4%) the CHD was judged to be hemodynamically significant. Thirty-one children of this cohort had been born prematurely, 9 of who were diagnosed with hemodynamically significant CHD. One child was diagnosed with chronic lung disease. Thus in the 2 cohorts combined, 180 of a total of 395 children with DS (45.6%) had ≥1 risk factor for severe RSV LRTI. In the control group, 14 (5.1%) of 276 siblings had been born prematurely and 3 children (1.1%) had CHD, which was hemodynamically significant in 1 (0.4%).

Incidence of RSV LRTI-Associated Hospitalization

The incidence of RSV LRTI-associated hospitalization was similar in both the retrospective and prospective cohort, including in children without known risk factors (8.1% versus 7.0%). In the combined cohort, 39 (9.9%) of 395 children with DS were hospitalized for RSV LRTI. (Fig 2) The cases of RSV LRTI-associated hospitalization were equally distributed in different age groups. (Table 2) The rate of hospitalization for RSV LRTI was lower in term children with DS without CHD (7.6%) than in preterm children with DS (9.4%) or in children with DS and significant CHD (11.9%), but these differences did not reach

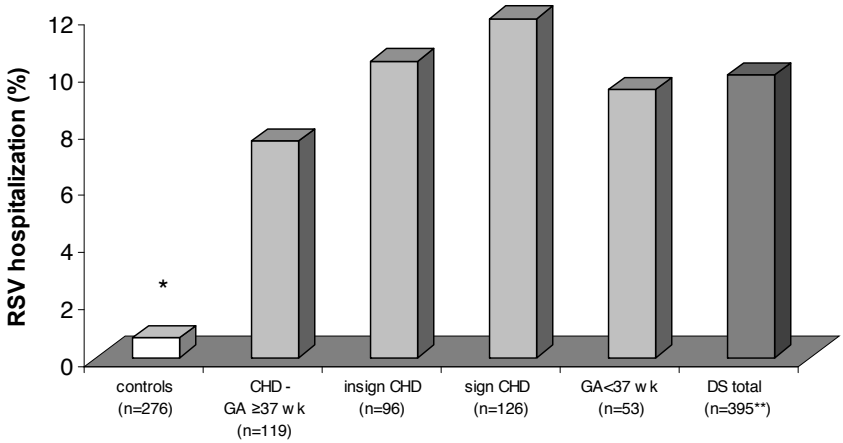


Figure 2. Incidence of RSV LRTI-associated hospitalization in children with Down syndrome

GA, gestational age; wk, weeks; CHD-, no diagnosis of congenital heart disease; sign CHD, diagnosis of hemodynamically significant congenital heart disease present; insign CHD, diagnosis of hemodynamically insignificant congenital heart disease present; DS, Down syndrome

* $p<0.001$ for controls versus each other category shown

** Includes one patient with chronic lung disease as a risk factor for severe RSV infection.

Table 2. Age at time of RSV LRTI-associated hospitalization

Age *, mo	Retrospective, n	Prospective, n	Total, n
0 – 3	4	8	12
3 – 6	3	5	8
6 – 12	5	6	11
12 – 24	4	4	8

* Age indicates age at time of diagnosis of RSV LRTI

statistical significance. Three children with hemodynamically significant CHD had had complete surgical correction of their heart defect ≥6 weeks before RSV LRTI-associated hospitalization. Two healthy children (0.7%) from the control group were hospitalized for RSV LRTI. Term children with DS without a heart defect were compared with the control group. Because control children did not have routine echocardiography at birth and might have had undiagnosed insignificant CHD, we combined the group of children with DS with hemodynamically insignificant CHD and the group of children with DS without CHD to calculate the odds ratio (OR) for RSV LRTI-associated hospitalization. The OR for RSV LRTI-associated hospitalization was 12.6 (95% confidence interval: 2.9-54.5) among term children with DS without hemodynamically significant CHD and 10.5 (95% confidence interval: 2.2-49.5) among term children with DS without any CHD.

Disease Severity and RSV LRTI-Associated Hospitalization

The severity of RSV LRTI was scored on the basis of the duration of hospitalization, need for supplemental oxygen, and need for mechanical ventilation. The characteristics of RSV LRTI-associated hospitalization are shown in Table 3. Disease severity was not significantly different between children with and without additional risk factors. The 39

Table 3. Characteristics of RSV LRTI-associated hospitalization in children with Down syndrome

	Retrospective study			Prospective study			Total of both studies		
	preterm	CHD	no RF	preterm	CHD	no RF	preterm	CHD	no RF
Total patients	5	4	7	0	11	12	5	15	19
Male	4 (80)	3 (75)	6 (86)	0	5 (46)	6 (50)	4 (80)	8(53)	12 (63)
Age *	9 (1-20)	8 (3-25)	3 (0.5-19)	0	4(0.5-21)	6 (2-18)	9 (1-20)	5(0.5-25)	5(0.5-19)
Severe co-morbidity	0	1 (25)	1 (14)	0	1 (9)	1 (8)	0	2(13)	2 (11)
Duration of hospitalization **	7 (4-49)	14 (4-34)	10 (6-28)	0	10 (2-77)	9 (2-65)	7 (4-49)	10 (2-77)	9 (2-65)
Supplemental oxygen	5 (100)	3 (75)	4 (57)	0	9 (82)	10 (83)	5 (100)	12 (80)	14 (74)
Mechanical ventilation	1 (20)	1 (25)	0	0	2 (18)	1 (8)	1 (20)	3 (20)	1 (5)

The number of patients is shown with percentages in parentheses unless otherwise specified.

* median age at time of diagnosis of RSV LRTI in months is given with range in parentheses

** median duration of RSV LRTI-associated hospitalization is shown in days with range in parentheses

CHD indicates children with significant congenital heart disease; RF indicates risk factor

children of the combined cohort with DS, who required hospitalization for RSV LRTI, had a median duration of hospitalization of 10 days; 31 children (79.5%) required supplemental oxygen; 5 children (12.8%) required mechanical ventilation.

DISCUSSION

We found a high incidence of RSV LRTI-associated hospitalization among children with DS. Although pediatricians are keenly aware of the high risk of infections among individuals with DS, [10;12] to our knowledge this is the first report of an increased incidence of RSV LRTI-associated hospitalization among children with DS, namely, 9.9% vs 0.7% in control children or 0.5 to 2% among the general pediatric population. [1]

Different pathophysiologic mechanisms could underlie the high risk of RSV LRTI-associated hospitalization seen among children with DS. For example, ~50% of children with DS have CHD, [7] and hemodynamically significant CHD is likely to be a risk factor for severe RSV LRTI in children with DS. However, no studies have been performed to establish the risk of severe RSV LRTI-associated hospitalization in children with DS-related CHD. In literature, pulmonary hypertension has been mentioned as a possible risk factor for severe RSV infection. [13] Pulmonary vascular resistance hypertension occurs more often in children with DS than in the general pediatric population. [14;15] Shah et al [16] reported that 10% of children with DS had pulmonary hypertension in the absence of CHD. In the current study, pulmonary hypertension was not evaluated in detail. Future studies will have to determine the role of pulmonary hypertension in severe RSV infection in children with DS. Children with DS have an abnormal upper airway physiology, which makes them prone to apnea. [17;18] It is possible that apnea is triggered by infections with respiratory viruses, especially RSV. In addition, a decreased lung function at birth may be important in the pathogenesis of RSV LRTI-associated hospitalization. [6] Lastly, children with DS seem to have an altered immune response, with thymus development and function being abnormal. [19-21] The number of B cells and T cells are low, especially in the first 2 years of life. [22] In addition, the defective T-cell *ex vivo* proliferative responses to nonspecific and antigenic stimuli, cytokine production, and natural killer cell responses detected in individuals with DS are thought to be important to the increased susceptibility of these individuals to infectious pathogens. Taken together, abnormal innate and adaptive immune responses in infants with DS could predispose them to severe disease if they become infected with RSV.

This study has potential methodological limitations. CHD is a known risk factor for RSV LRTI-associated hospitalization. [4] However, although 63% of our population had CHD, only 35.7% had hemodynamically significant CHD. Moreover, the power of this study was inadequate to show differences in the incidence of RSV LRTI-associated hos-

pitalization or disease severity among subgroups of children with DS. We consider it plausible that hemodynamically significant CHD and prematurity are independent risk factors for RSV LRTI-associated hospitalization in children with DS. We did not have a matched-control group, but instead collected information from siblings. The rate of RSV LRTI-associated hospitalization of 0.7% in this large control group was consistent with estimates reported in the literature, which supports the estimated OR of 12.6 for RSV LRTI-associated hospitalization in children with DS without known risk factors for RSV LRTI. [1]

It could be argued that hospitalization bias explains our study results. However, we do not think that the hospitalization threshold for RSV LRTI was lower for the children with DS. In fact, the children with DS may have had more severe RSV LRTI than the general pediatric population. The duration of hospitalization for RSV LRTI varies among countries, but in the Netherlands the median length of stay is 8 days. [2] It was 10 days in the children with DS. Moreover, 79.5% of the hospitalized children with DS required supplemental oxygen, compared with 38.7–68.2%, and 12.8% of the children with DS required mechanical ventilation, compared with 2.2% in the general pediatric population. [2] These results indicate that the children with DS had more severe RSV LRTI and that the increased incidence of RSV LRTI-associated hospitalization was not because of hospitalization bias.

The results may have clinical implications. We found a substantial proportion of children with DS to have RSV LRTI. Although vaccination against RSV LRTI is not yet possible, targeted RSV prophylaxis in high-risk populations is currently possible with palivizumab, a monoclonal antibody against the F-protein of RSV. [23] We also found DS to be associated with a similar rate of RSV LRTI-associated hospitalization as that previously reported for prematurity, chronic lung disease, and CHD. [13;23;24] Our findings therefore support the possibility of a new indication for RSV prophylaxis in children with DS up to 2 years of age, although the safety and efficacy of such an approach remains to be determined.

CONCLUSIONS

Our study demonstrates a very high incidence of RSV LRTI-associated hospitalization in children with DS and suggests that these children have more severe disease when hospitalized. These findings warrant additional study of the mechanisms underlying severe RSV LRTI in children with DS.

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Les Ruinettes (2227m)
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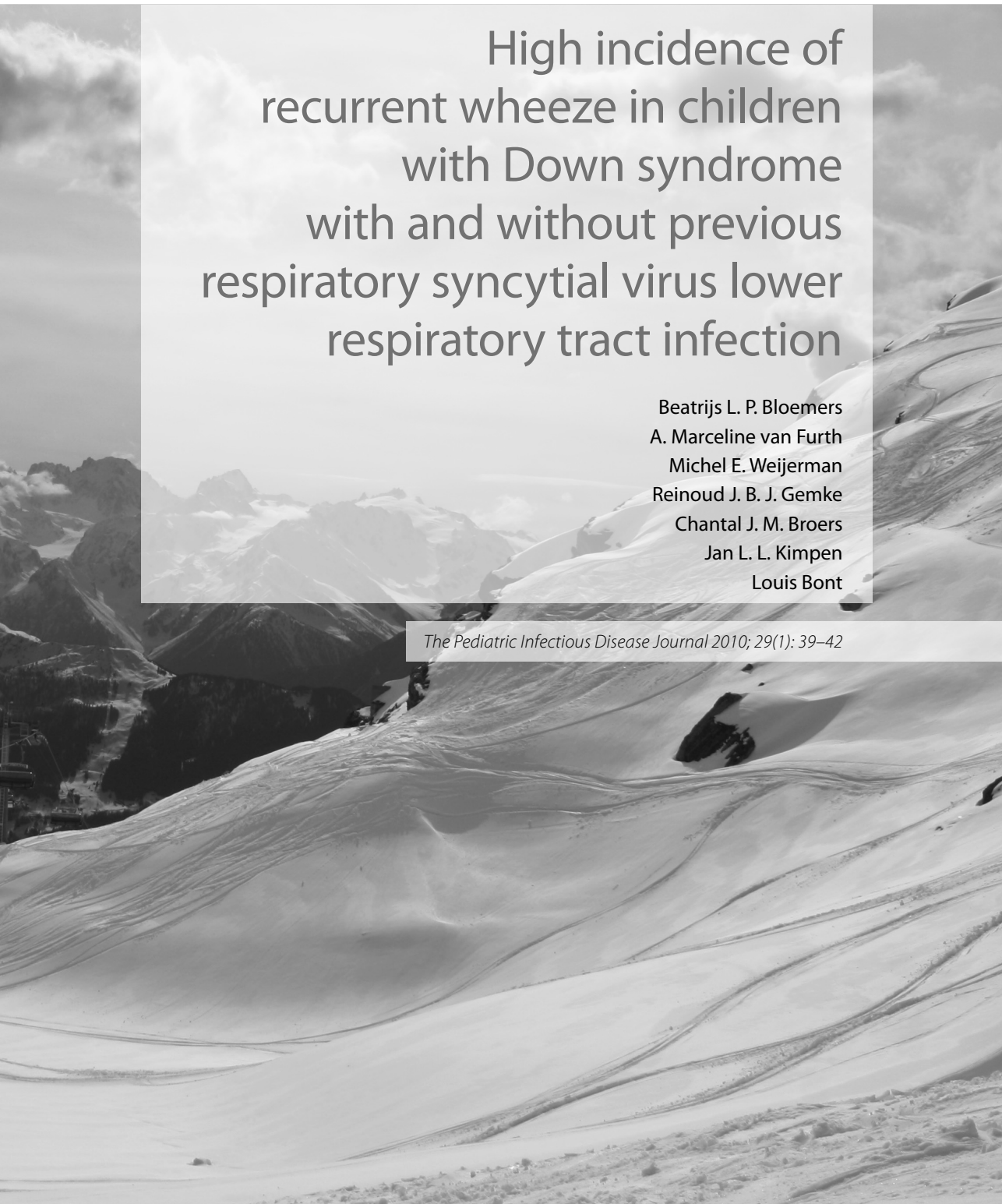
Chapter 6



High incidence of recurrent wheeze in children with Down syndrome with and without previous respiratory syncytial virus lower respiratory tract infection

Beatrijs L. P. Bloemers
A. Marceline van Furth
Michel E. Weijerman
Reinoud J. B. J. Gemke
Chantal J. M. Broers
Jan L. L. Kimpen
Louis Bont

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ABSTRACT

Objectives

Respiratory syncytial virus (RSV)-induced lower respiratory tract infection (LRTI) is associated with the subsequent development of recurrent wheeze. In a recent study, we found a high incidence of 9.9% of hospitalization for RSV-induced LRTI among children with Down syndrome (DS), indicating DS as a new risk factor for RSV-induced LRTI. In the current study we aimed to investigate the development of long-term airway morbidity in children with DS following hospitalization for RSV-induced LRTI.

Methods

A combined retrospective cohort and prospective birth cohort of children with DS with a history of hospitalization for RSV-induced LRTI was studied (n=53). Three control populations were included: children with DS without hospitalization for RSV-induced LRTI (n=110), children without DS but with hospitalization for RSV-induced LRTI (n=48), and healthy siblings of the previous three groups mentioned (n=49). The primary outcome was physician-diagnosed wheeze up to two years of age.

Results

The incidence of physician-diagnosed recurrent wheeze in children with DS with a history of hospitalization for RSV-induced LRTI was 36%. Unexpectedly, up to 30% of children with DS without a history of RSV-induced LRTI had physician-diagnosed recurrent wheeze (no significant difference). In children without DS physician-diagnosed wheeze was found more frequently in children hospitalized for RSV-induced LRTI than healthy controls (31% vs 8%, p=0.004).

Conclusions

In this combined retrospective/prospective cohort study RSV-induced LRTI did not significantly contribute to the risk of recurrent wheeze in children with DS. An unexpected finding was that recurrent wheeze was very common among children with DS.

INTRODUCTION

Respiratory syncytial virus (RSV) is the single most important cause of lower respiratory tract infections (LRTIs) in infants and young children. [1] About 0.5–2.0% of infected children require hospitalization, but children with Down syndrome (DS) have an increased risk of being hospitalized for RSV-induced LRTI (9.9%). [2] Between 41% and 72% of young children experience recurrent episodes of wheezing following RSV-induced LRTI [3-6]; however, to our knowledge recurrent wheeze in children with DS has not been previously studied.

Studies of the pathophysiology of recurrent wheeze following RSV-induced bronchiolitis and the association with asthma in later life have yielded contradictory findings. Most studies show a transient relationship between RSV-induced bronchiolitis and recurrent wheeze, with wheeze no longer being associated with a history of RSV-induced bronchiolitis in school-age children. [7-11] However, a few reports suggest that RSV is causally associated with the development of persistent allergic asthma. [12;13] In a recent study, we established that DS is an independent risk factor for hospitalization for RSV-induced LRTI and that children with DS also tended to have more severe disease. [2] The aim of this study was to determine the incidence of recurrent wheeze following RSV-induced LRTI in children with DS.

METHODS

Study population

We included a retrospective cohort of children with DS with a history of hospitalization for RSV-induced LRTI (n=30), who were being monitored by the Down Syndrome Study Group. These children, born between 1988 and 2007, attended the outpatient clinic of the pediatric department of either the VU University Medical Centre Amsterdam or the University Medical Centre Utrecht. Subsequently, a prospective longitudinal birth cohort study of children with DS, born between 2003 and 2005 was included as described in our previous paper. [2] This birth cohort consisted of 23 children with DS who had been hospitalized for RSV-induced LRTI. Thus, the total index group consisted of 53 children with DS who had been hospitalized for RSV-induced LRTI (DS+ RSV+). Three control groups were included, to differentiate between DS and hospitalization for RSV-induced LRTI as risk factors for recurrent wheeze. (Figure 1) The first control group (n=110) consisted of children with DS from the same birth cohort as part of the index group, but without a history of hospitalization for RSV-induced LRTI (DS+ RSV-). The second control group (n=48) consisted of children without DS who had been hospitalized for RSV-induced LRTI (DS- RSV+). These children were selected from the placebo group

of a trial on corticosteroid use for RSV-induced LRTI. [14] A third control group (n=49) of healthy controls consisted of siblings (aged 1–4 years) of the children from the other three groups studied (DS- RSV-). Children with hospitalization for RSV-induced LRTI had either a positive enzyme immunoassay for RSV, a positive direct immunofluorescence assay for RSV infection of epithelial cells in nasopharyngeal secretions or a positive viral culture for RSV.

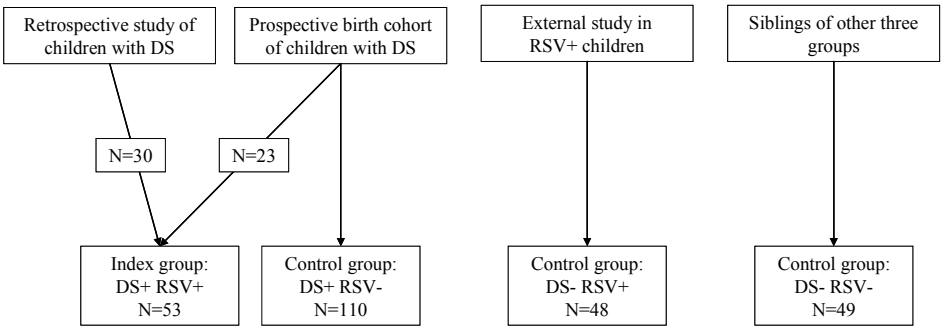

Figure 1. Selection of children in the analysis

Data collection

A short standardized questionnaire was taken by one investigator (BB) from the primary physician to inquire whether signs of airflow limitation had been noted upon physical examination and mentioned in the patient's chart in at least two independent consultations or whether the child had ever been diagnosed with asthma. Secondly, parents were asked to complete a questionnaire on their children's health in the first two years after hospitalization for RSV-induced LRTI or up to the age of two years in the case of controls who had not been hospitalized for RSV-induced LRTI. Extended Dutch versions of the standardized British Medical Council questionnaire and the European Community Respiratory Health Survey questionnaire were used to obtain data on the presence and frequency of wheezing. [15-17] Information on confounding factors, such as parental smoking habits, a family history of atopic symptoms, gestational age, and number of siblings, was also collected. The primary outcome was physician-diagnosed recurrent wheeze. Secondary outcomes were parent-reported recurrent wheeze and physician-diagnosed asthma. Recurrent wheeze was defined as two or more separate episodes of wheeze in a period of two years. It was not studied how a diagnosis of asthma was made at this young age. Written parental informed consent was obtained for the collection of all data.

Statistical analysis

Differences in baseline characteristics were compared using Chi-square. The percentage of physician-diagnosed recurrent wheeze or asthma or parent-reported recurrent wheeze was compared using Chi-square analysis. For children with DS, logistic regression analysis was performed to evaluate the independent determinants of recurrent wheeze or physician diagnosed asthma, including hospitalization for RSV-induced LRTI, sex, prematurity, patient eczema, number of siblings, parental smoking and parental history of atopy. All statistical analyses were performed using the software program SPSS for Windows (version 12.0.2; SPSS Inc., Chicago, IL). A *P* value of .05 was considered the limit of significance.

RESULTS

Study Population

Fifty-three children with DS who had been hospitalized for RSV-induced LRTI were compared with children with DS without RSV-induced LRTI (n=110), children without DS who had been hospitalized for RSV-induced LRTI (n=48), and healthy controls (n=49). Baseline characteristics and risk factors for recurrent wheeze are given in Table 1. There were no significant differences in baseline risk factors for recurrent wheeze between the prospective and retrospective cohort of the index group (data not shown). The two cohorts were combined as one index group. Median age of hospitalization for RSV-induced LRTI in children with and without DS was similar (6 versus 4.5 months, not significant). The significant difference in the presence of one or more siblings at home in the healthy

Table 1. Baseline characteristics and risk factors for recurrent wheeze

	DS+ RSV+	DS+ RSV-	DS- RSV+	DS- RSV-
	n=53	n=110	n=48	n=49
Male sex	32 (60%)	56 (51%)	22 (46%)	28 (57%)
Median age *	6 (0-27)		4,5 (0-12)	
Prematurity	9 (17%)	17 (15%)	7 (15%)	2 (4%)
Patient eczema **	5 (9%)	9 (8%)	9 (19%)	11 (22%)
Parental atopy	30 (57%)	55 (50%)	28 (58%)	24 (49%)
Parental smoking	10 (19%)	9 (8%)	4 (8%)	3 (6%)
Siblings at home ***	38 (72%)	82 (75%)	35 (73%)	46 (94%)

* at time of RSV hospitalization, months (range)

** $P < 0.05$ for DS- RSV- versus DS+ RSV-.

*** $P < 0.01$ for DS- RSV- versus all other groups.

DS indicates Down syndrome; RSV indicates respiratory syncytial virus.

control group was because this group was made up of the siblings of children in the other three groups.

RSV-induced LRTI does not have a significant effect on recurrent wheeze in DS

Physician-diagnosed recurrent wheeze in DS+RSV+ and DS+RSV- children was 36% and 30% respectively (not significant) (Figure 2). Similar non-significant differences were found for parent-reported recurrent wheeze (42% versus 32%) and physician-diagnosed asthma (11% versus 9%, data not shown). No significant differences in outcome were found between the retrospective and prospective cohort of DS+RSV+ children (data not shown). Apparently, hospitalization for RSV-induced LRTI did not significantly increase the risk of long-term respiratory tract morbidity of children with DS. In addition to recurrent wheeze parents reported comparable respiratory symptoms in DS+RSV+ and DS+RSV- children. The presence and frequency of symptoms of URTI did not differ between the two groups. Respiratory medication, such as bronchodilators and steroids, were prescribed slightly more often in DS+ RSV+ compared to DS+ RSV- children (72% versus 54%, p=0.04). In children without DS we confirmed that a history of RSV-induced LRTI increased the risk of physician-diagnosed recurrent wheeze (31% in DS- RSV+ versus 8% in DS- RSV-, p=0.004), parent-reported recurrent wheeze (56% versus 4%, p<0.001) and physician-diagnosed asthma (8% versus 0%, p=0.056, data not shown). Logistic regression analysis, including hospitalization for RSV-induced LRTI, sex, prematurity, patient eczema, number of siblings, parental smoking and parental history of atopy

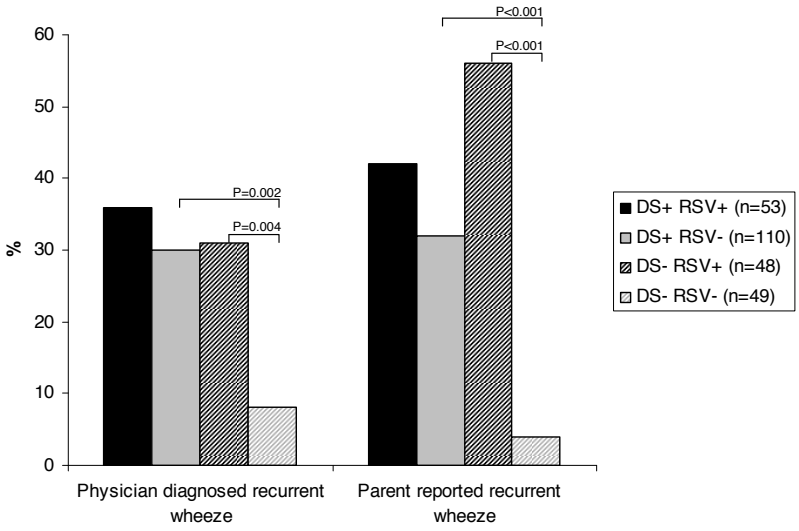

Figure 2. Incidence of recurrent wheeze

confirmed that hospitalization for RSV-induced LRTI altered the risk of recurrent wheeze or physician-diagnosed asthma only in children without DS, but not in children with DS (data not shown).

DISCUSSION

This study showed that hospitalization for RSV-induced LRTI in children with DS did not significantly increase the risk of long-term airway morbidity. The previously reported association between severe RSV-induced LRTI and the subsequent development of recurrent wheeze was confirmed in children without DS. [3-6] An unexpected finding was the generally high incidence of recurrent wheeze in children with DS, both with and without a history of severe RSV-induced LRTI.

To our knowledge, this is the first report on recurrent wheeze among children with DS. There have been a few studies of asthma in children with DS, and these report either a reduced incidence of asthma (0.2%) or a normal incidence of asthma (17%) with mild disease. [18-21] Although the methodologies of these studies were not described in detail, the findings suggest that DS might have a protective effect regarding the development of asthma. This is in apparent contrast with our findings, in which the incidence of recurrent wheeze was higher among children with DS, regardless of whether they had a history of RSV-induced LRTI, than among control children without DS. However, an increased incidence of wheeze before three years of age does not exclude a normal or decreased risk of allergic asthma at school age. [22;23]

The results of this study show that children with DS have a high risk of recurrent wheeze independent of a history of severe RSV-induced LRTI. Previously described factors associated with recurrent wheeze, such as family size, parental smoking and atopy, did not have a significant effect on the outcome of recurrent wheeze or asthma in children with DS, although this study was not powered to demonstrate more subtle effects. Different pathophysiological mechanisms could underlie the generally increased risk of recurrent wheeze among children with DS. First, airway physiology may be abnormal in children with DS: a high incidence of airway anomalies have been reported, with laryngomalacia and tracheomalacia being the most frequent endoscopic findings [24], and anomalies at a more distal site have been found in 75% of children with DS with diagnosed laryngomalacia. In addition, patients with DS show disturbed lung growth, including reduced lung volume, reduced airway generation, and excessive alveolar multiplication with a polyalveolar structure. [25] Secondly, there could be genetic factors which are related to both the risk of RSV-induced LRTI and recurrent wheeze. To our knowledge currently published candidate genes for RSV-induced LRTI and asthma are not located on chromosome 21. [22;26] However, a number of proteins that have a role in the immune system, such as CuZn-

superoxide dismutase, lymphocyte function-associated antigen, and the interferon receptor, are coded by genes located on chromosome 21. [27] For example, an increased expression of the CuZn-superoxide dismutase gene results in disorganization of the thymus with subsequent abnormal maturation of thymocytes, leading to functionally disturbed T lymphocytes. It would be interesting to determine whether these genes are associated with bronchiolitis or asthma. In addition to abnormal thymus development and function, children with DS have a low absolute number of B-cells and T-cells, especially in the first 2 years of life. The defective T-cell *ex vivo* proliferative responses to non-specific and antigenic stimuli, cytokine production, and NK-cell responses detected in individuals with DS are thought to be involved in the increased susceptibility of these individuals to infectious pathogens. [28;29] In our study, all the wheezing episodes reported by the parents occurred in combination with a common cold. Therefore, the high incidence of RSV-induced LRTI and recurrent wheeze in children with DS could reflect a generally defective defense against respiratory viral pathogens. In conclusion we hypothesize that children with DS have a combination of pre-existent lung abnormalities, genetic factors and immunologic deficits that make them more susceptible to respiratory viruses and subsequently results in a high incidence of both RSV LRTI and recurrent wheeze in a parallel manner rather than a serial one.

This study has potential methodologic limitations. First, this study was not powered to detect small, but relevant effects of RSV-induced LRTI on recurrent wheeze. However, it is emphasized that there were virtually no differences in outcome between children with DS with and without RSV-induced LRTI. Second, parent-reported wheeze might be biased because of the subjectivity of reporting symptoms. Recall bias could play a role in the high incidence of recurrent wheeze in the group of children without DS who had been hospitalized for RSV-induced LRTI because the parents of these children had kept a daily log on respiratory symptoms for a different study. For that reason we used physician diagnosed wheeze as primary outcome, being based on written information from the patient's chart and therefore not prone to recall bias. Third, we can not exclude the possibility that the high incidence of recurrent wheeze in children with DS without hospitalization for RSV-induced LRTI is related to a history of RSV-induced LRTI which did not require hospital admission. Finally, we limited the study to children up to 2 years of age and thus can say nothing about the persistence of wheezing. Prolonged follow-up of children with DS aged 6–10 years may provide more insight into the long-term respiratory prognosis of children with DS who have been hospitalized for RSV-induced LRTI.

In conclusion, RSV-induced LRTI does not have a significant effect on the incidence of recurrent wheeze in children with DS. An unexpected finding was the high incidence of recurrent wheeze in children with DS without a history of severe RSV-induced LRTI. It is conceivable that the high incidence of recurrent wheeze and the high incidence

of hospitalization for RSV-induced LRTI in children with DS have a common etiology. Abnormal lung function or airway hyper responsiveness, as well as abnormal immunological maturation, could play a decisive role in the development of long-term airway morbidity in children with DS. Our results prompt future studies on lung development and immunology in DS to give better insight in the pathophysiologic mechanism of RSV LRTI and recurrent wheeze in this specific population.

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Chapter 7

General Discussion

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OBJECTIVES AND MAIN FINDINGS

The aim of this thesis was to add to our understanding of the function of the immune system in children with Down syndrome (DS) and to determine the burden of respiratory tract infections, in particular respiratory syncytial virus (RSV) infections, and its long term consequence of recurrent wheeze. The main findings of this thesis will be discussed and suggestions are made to further improve the medical care of children with DS.

Our main findings are:

1. Children with DS show distinct abnormalities of cells of the innate immune system, most strikingly an increased frequency of pro-inflammatory $CD14^{dim}CD16^{+}$ monocytes.
2. Decreased thymic output but not disturbed peripheral dynamics explains decreased naive T-cell numbers in children with DS.
3. Down syndrome is an independent risk factor for RSV associated hospitalization.
4. Children with DS have a high prevalence of recurrent wheeze, irrespective of RSV associated hospitalization.

IMMUNOLOGY IN CHILDREN WITH DS

Distinct abnormalities in the innate immunity of children with DS: cause or consequence?

Over the years innate immunity has not been given much attention in DS immunologic research. One aim of this thesis was to study frequency of different cell types of in particular the innate immune system in the blood of children with DS. Using the currently accepted definitions of innate immune cells we have performed cell surface immunophenotyping of natural killer (NK) cells, invariant natural killer T-cells (iNKT), dendritic cell and monocyte subsets *(Chapter 3)*. As with most leukocyte subsets, children with DS showed lower absolute numbers of almost all innate immune cell subsets as compared to healthy controls. This finding might suggest general dysfunction of the bone marrow. A remarkable finding was the significantly higher absolute number of non-classical $CD14^{dim}CD16^{+}$ monocytes in children with DS, while the classical $CD14^{+}CD16^{-}$ monocytes were decreased compared to healthy controls. Increased numbers of $CD14^{dim}CD16^{+}$ monocytes have been described in different acute and chronic inflammatory diseases like sepsis, HIV and malignancies. [1-4] These diseases clearly have a distinct pathogenesis. Therefore, high numbers of $CD14^{dim}CD16^{+}$ monocytes may be the consequence, rather than a cause of chronic inflammation. The most important argument against this hypothesis in children with DS is that children with DS in our study appeared healthy. However, subclinical

chronic inflammation cannot be excluded at this point. For example, unrecognized auto-immune disease in the gastrointestinal mucosa could have been present in some children with DS. Alternatively, increased numbers of $CD14^{dim}CD16^{+}$ monocytes may be the actual cause of the high morbidity, playing a role in the susceptibility to auto-immune diseases in individuals with DS later in life. We have no follow-up data of our cohort to establish whether children with the highest numbers of $CD14^{dim}CD16^{+}$ cells would subsequently develop auto-immune disease. Future longitudinal studies of immune parameters related to outcome of (auto-immune) disease are needed to clarify this question.

T-cells in children with DS: the thymus revisited

Several studies in children with DS have shown low numbers and disturbed function of T-cells. [5-13] In *Chapter 2* we reviewed this literature, which suggests that abnormal thymic development and function might cause these T-cell abnormalities in children with DS. [14-19] Our study on the dynamics of the naive T-cell compartment (*Chapter 4*) has confirmed and expanded previous findings of low absolute numbers of naive T-cells in children

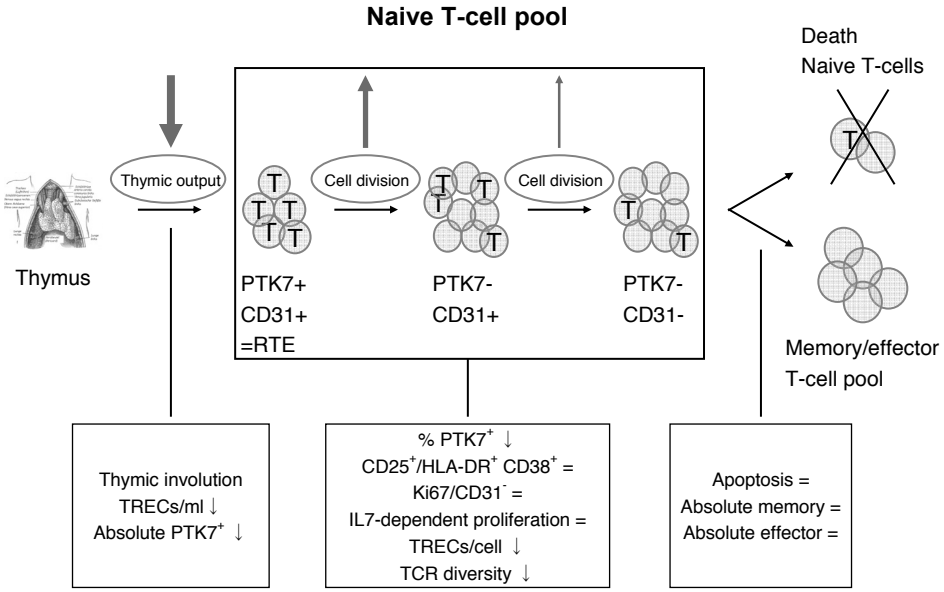

Figure 1. Dynamics of the naive T-cell pool in children with DS

The results of our study on the dynamics of the naive T-cell pool are shown in figure 1. Thymic output of recent thymic emigrants (RTE), peripheral expansion and longevity of existing naive T-cells have a positive effect on the number of naive T-cells in the peripheral blood. Apoptosis and clonal expansion to a memory or effector phenotype upon antigen stimulation decrease the number of cells in the naive T-cell pool. The findings are summarized below the figure showing a decreased input of RTE, a normal to increased peripheral generation of naive T-cells and a normal loss of naive T-cells through apoptosis and clonal expansion.

T, a TREC containing T-cell; TRECs, T-cell receptor excision circles; PTK7, protein tyrosine kinase 7 positive or negative naive T-cells; TCR, T-cell receptor.

with DS. The establishment and maintenance of the naive T-cell pool is a dynamic process. [20;21] Consequently, it is not possible to conclude thymic insufficiency based on the number of naive T-cells detected in the peripheral blood. [22] Figure 1 shows the balance of the naive T-cell pool and summarizes our findings on this subject in children with DS. The absolute number of naive T-cells is influenced by input of cells (production) and by output of cells (use or loss). Production of naive T-cells is primarily the role of the thymus that releases these cells in the peripheral blood. [23] However, survival and peripheral proliferation of naive T-cells without loss of phenotype result in maintenance of the naive T-cell pool as well. [20;21] Output from the naive T-cell pool is established by differentiation into a memory phenotype upon antigen encounter or by death through apoptosis. In healthy subjects, the number of naive T-cells remains rather stable up to the age of 65 years. [24-26] We found that maintenance of the naive T-cell pool is disturbed in children with DS, which is mainly caused by decreased thymic output. Survival or peripheral proliferation is normal or even slightly increased, suggesting compensation for the decreased input of naive T-cells by the thymus. No evidence for enhanced naive T-cell loss through apoptosis or differentiation to an effector/memory pool was found in children with DS. We conclude that abnormal thymic development causes low number of naive T-cells in children with DS, but peripheral mechanisms regulating the size of the naive T-cell pool appear intact.

RSV AND RECURRENT WHEEZE IN CHILDREN WITH DS

Epidemiology

We performed a prospective birth cohort study to identify DS as a new, independent risk factor for RSV lower respiratory tract infection (LRTI) associated hospitalization (*Chapter 5*). Although this finding might not have surprised many clinicians, no prospective study had been performed in children with DS. Within children with congenital heart disease (CHD) however it has been previously reported and recently confirmed that children with co-morbidity of DS have a higher risk of RSV associated hospitalization. [27;28] In our combined retrospective and prospective birth-cohort of 395 children with DS we have shown an incidence of 9.9% of hospitalization for RSV LRTI. This was in contrast to control children of which only 0.7% had been hospitalized. Since more than half of the children with DS had either significant (n=126) or insignificant CHD (n=96), separate ORs were calculated for term children with DS without hemodynamically significant CHD (OR 12.6; 95% confidence interval (CI): 2.9-54.5) and for term children with DS without any CHD (OR 10.5; 95% CI: 2.2-49.5). The focus of this thesis was on RSV-specific LRTI in children with DS, but unpublished parental reports on high frequency of hospitalization

for LRTIs caused by other pathogens, suggest that our result may also be applicable to other viruses, e.g. rhinovirus, (para)influenza-virus, adenovirus or others as well.

Previous prospective studies have shown that severe RSV infection is associated with recurrent wheeze during childhood. [29] The prevalence is highest in the first year following hospitalization and decreases during subsequent years. [30-33] Distinct clinical, genetic and immunological determinants of early and late wheeze following RSV associated hospitalization have been identified. [30;34-36] As a result, early wheeze following RSV associated hospitalization is distinct from allergic asthma. [35] We found a high incidence of recurrent wheeze in children with DS in the first two years of life (*Chapter 6*). Children with DS had recurrent wheeze in 36% of cases after RSV associated hospitalization, which was comparable to control children with RSV associated hospitalization but without DS (31%). A remarkable finding was the high incidence of recurrent wheeze in children with DS without a history of severe RSV (30%). This result appeared to contradict with data suggesting that children with DS have a decreased incidence of atopic diseases, such as asthma and allergic rhinitis. [37-41] However, in the context of early and late wheeze being distinct entities [35;36], we showed a high incidence of early recurrent wheeze, which probably reflects viral induced wheeze. Our study did not provide any information on late wheeze and therefore does not exclude the possibility that children with DS have a normal or even a decreased risk of allergic asthma.

In conclusion, we have described DS as a new risk factor for RSV-associated hospitalization. This finding might reflect a general susceptibility of children with DS to respiratory tract infections (RTIs), which is suggested by the high incidence of recurrent wheeze irrespective of a history of RSV associated hospitalization.

Pathophysiology

Parallel predisposition

The finding of a high incidence of both RSV associated hospitalization and recurrent wheeze irrespective of RSV suggests a general disturbance in viral clearance rather than a RSV-specific problem in children with DS. Although we only studied RSV LRTI associated hospitalization in detail, 8.6% of children with DS were hospitalized for RTIs caused by other pathogens than RSV (unpublished data). Different pathophysiologic mechanisms present in DS might predispose these children in general to viral RTIs which results in our findings of a high incidence of both RSV-infections and recurrent wheeze in a primary parallel way. Therefore, it is concluded that RSV does not add to the prevalence of recurrent wheeze. Our proposed model of parallel predisposition is shown in Figure 2.

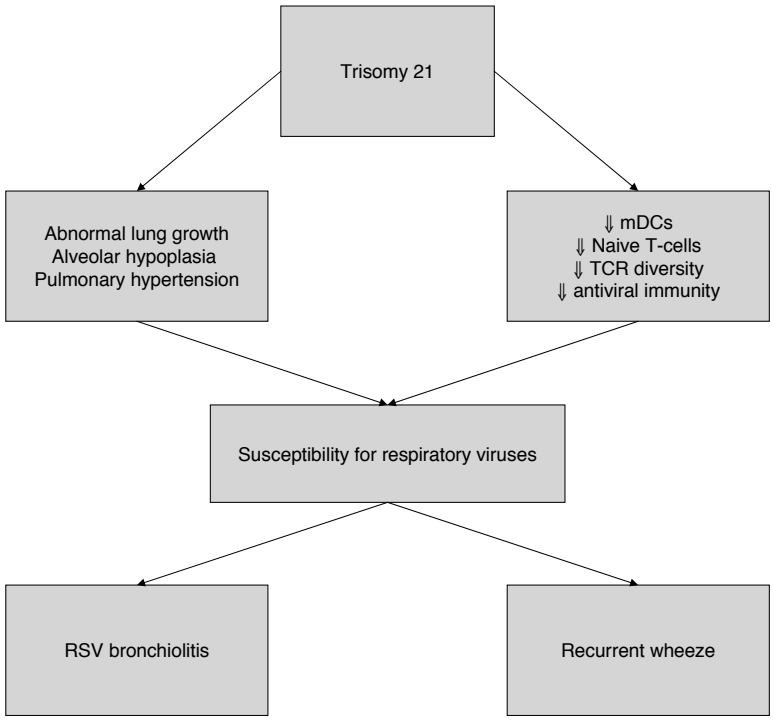

Figure 2. Parallel predisposition in children with DS

Disturbed lung growth, alveolar hypoplasia and pulmonary hypertension might all contribute to disturbed airway physiology, which may result in decreased viral clearance and consequently a higher susceptibility to viral respiratory tract infections. A quantitative defect of both innate (mDCs) and adaptive immune cells (naive T-cells) has been shown in our studies in children with DS. In combination with loss of TCR diversity and previous literature showing qualitative dysfunction of DS immune cells resulting in decreased chemotaxis, NK-cell activity, and cytotoxicity, an important role for the immune system in decreased viral clearance in children with DS is suggested. It is conceivable that loss of immune cells both in quantity and quality will result in a higher predisposition to severe respiratory tract infections. Due to the combination of a disturbed airway physiology and a general diminished immune response, children with DS are predisposed to a high incidence of both RSV LRTI associated hospitalization and recurrent wheeze induced not specifically by RSV, but by any viral pathogen.

mDCs- myeloid dendritic cells; TCR- T-cell receptor

Abnormal lung development and primary pulmonary hypertension

As described in *Chapter 5 and 6*, a high frequency of airway anomalies such as laryngo- and tracheomalacia has been described in individuals with DS. [42] In combination with disturbed lung growth resulting in reduced lung volume and alveolar hypoplasia, these abnormalities may lead to a different airway physiology in children with DS compared to controls. [43]

Congenital heart disease is diagnosed in approximately 50-55% of children with DS. [44-46] In the seventies and eighties these children did not undergo corrective surgery at all or not until the age of 2-4 years, often resulting in pulmonary vascular resistance hyperten-

sion. [47] Pulmonary hypertension has been suggested in literature as a possible risk factor for severe RSV infection. [48] Surgical treatment has improved tremendously over the last two decades, which has almost eradicated pulmonary hypertension in these children with congenital heart disease. [49] However, pulmonary hypertension has been described in 10% of children with DS in the absence of congenital heart disease. [50] Disturbed lung growth, alveolar hypoplasia and pulmonary hypertension might all lead to disturbed airway physiology, which may result in decreased viral clearance and consequently a higher susceptibility to viral respiratory tract infections. Eventually this abnormal lung predisposition could lead to a higher risk of both RSV associated hospitalization and recurrent wheeze induced not specifically by RSV, but by any viral pathogen. A birth cohort study of children with DS with and without congenital heart disease in which pulmonary hypertension and lung function at different time points in the first two years are measured and subsequently related to clinical data of hospitalization for RSV or other severe respiratory tract infections, could give better insight in this issue.

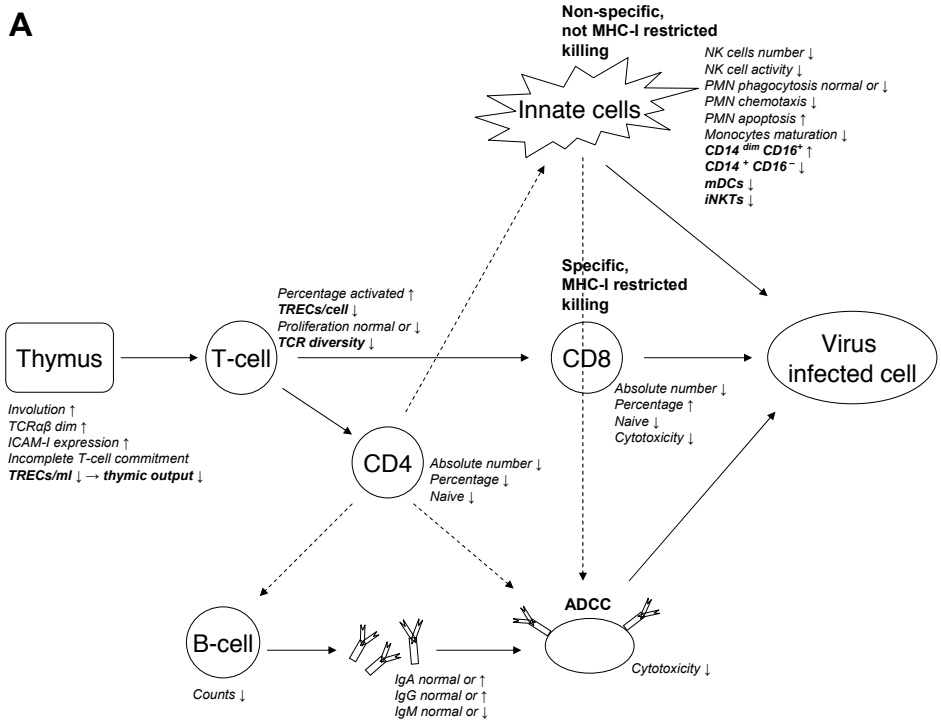

Figure 3. The role of immunologic abnormalities in pathogenic clearance in children with DS (Continued on next page)

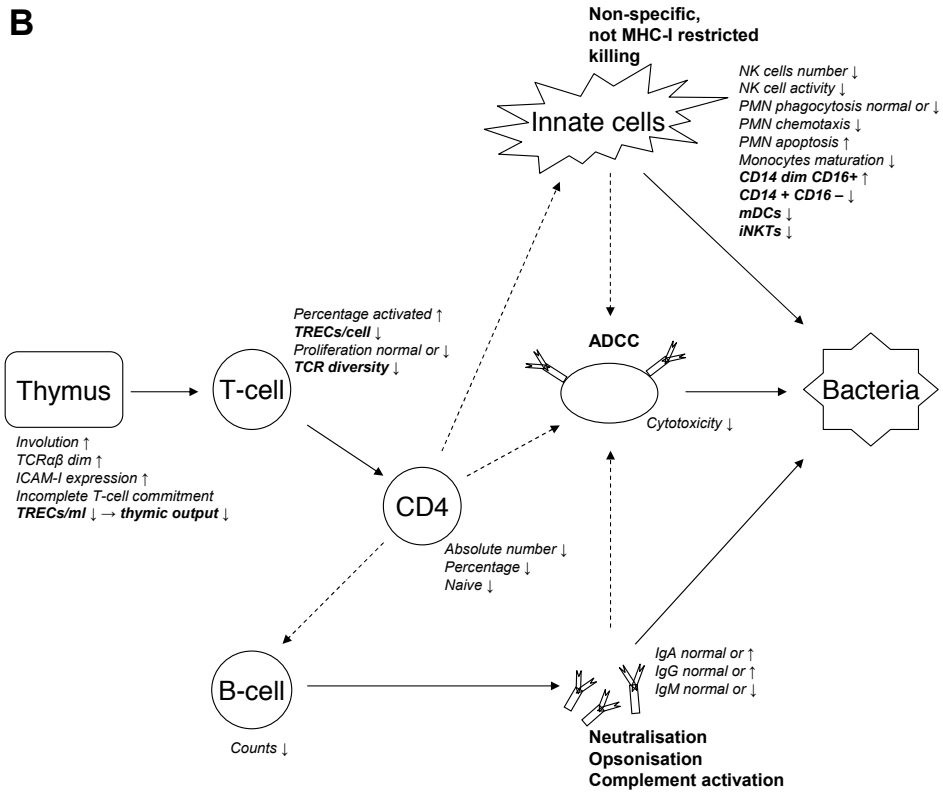

Figure 3. The role of immunologic abnormalities in pathogenic clearance in children with DS

The role of different immune cells in clearance of virus infected cells is shown in figure 3A. Viral immunity can be roughly divided in non-specific, not MHC-I restricted killing by innate cells (upper part of figure), specific, MHC-1 restricted killing by $CD8^+$ cytotoxic T-cells (middle part of figure) and antibody-dependent cell-mediated cytotoxicity (lower part of figure). Next to each cell type differences in number and function are summarized for children with DS. Figure 3B provides a summary of differences in cell types in individuals with DS that play a role in bacterial defense: not MHC-I restricted killing by innate cells (upper part of figure), antibody-dependent cell-mediated cytotoxicity (ADCC) (middle part of figure) and neutralization, opsonization and complement activation (lower part of figure).

The role of aberrant immunology in the pathogenesis of severe RSV infections and recurrent wheeze in children with DS

In addition to previous studies on immunology in children with DS, we have shown a variety of immunologic differences in this population compared to control children, that could play a role in the increased incidence of both RSV associated hospitalization and recurrent wheeze. In *Chapter 2* the literature on immunology in individuals with DS is reviewed and a model of disturbed viral clearance is proposed. This model can be adjusted upon the findings in this thesis (Figure 3). It is known from children with DiGeorge syndrome that low numbers of immune cells can lead to a higher susceptibility to infections. [51] In children with DS we showed decreased numbers of immune

cells, especially mDCs and naive T-cells, although not as low as in those children with severe immunodeficiency. It is not clear whether the decreased numbers of immune cells result in decreased capacity to clear viral pathogens in children with DS. Even in healthy subjects a broad range is used to define reference values, and still a clear cut off point at which an effective immune response is either maintained or lost cannot be provided. [52] In addition, as with most human clinical studies, we only describe cell counts in peripheral blood, without information on numbers of immune cells in peripheral tissues such as the lungs, i.e. the site of inflammation in case of respiratory tract infections. The children with DS studied in this thesis did not show any signs or symptoms of disease at time of blood collection and therefore we believe that numbers in peripheral blood are sufficient to draw first conclusions and to direct future research.

In our study on T-cell homeostasis in children with DS (*Chapter 4*) we showed that quantity of T-cells is the main issue in individuals with DS. Normal or even increased peripheral generation of the naive T-cell compartment was found, suggesting a normal function of these naive T-cells. We hypothesized that a possible increased peripheral generation reflects an attempt of the body to maintain normal cell numbers, although this cannot be fully reached in children with DS. Peripheral generation of naive T-cells can be induced by IL-7 through the IL-7-receptor, [53] and T-cell receptor (TCR)-major histocompatibility (MHC)/selfpeptide ligand interactions. [54;55] Within the naive T-cell compartment, IL-7 preferentially induces proliferation of the CD31 positive subset without changing its phenotype, while TCR-major histocompatibility (MHC)/selfpeptide ligand interactions are suggested to result in loss of CD31 expression. [56;57] A small, but not significant, increase in $CD31^-$ naive $CD4^+$ T-cells was shown in our study, but with normal IL-7 dependent proliferation of naive T-cells. TCR-driven peripheral expansion results in clonal expansion of naive T-cells expressing high affinity TCRs and as a consequence loss of TCR diversity is seen. [57] A significant increase in number of oligoclones of the TCR Vβ family was shown in children with DS compared to controls. A diverse repertoire of TCRs gives a host the opportunity to recognize a wide range of pathogens. Loss of TCR diversity might therefore result in decreased viral clearance in children with DS.

Although multiple immunologic abnormalities have been described in individuals with DS by us and many others, it is difficult to state whether these abnormalities are the cause or consequence of the high morbidity. In the last two decades a mouse-model of DS has been developed, which is mostly used for Alzheimer's studies of DS. [58] It would be of interest to investigate whether this model is adequate for basic immunologic studies as well, since only part of chromosome 16 (the mouse analogue of human chromosome 21) is inserted in these mice. If this model is a good representation of the human DS immune system, different studies could be designed that examine the immune system before and after the mouse has been infected with RSV and other respiratory viruses.

The development of the immune system can be examined and the influence of acute and chronic infections on the distribution of different cell subsets determined.

In short, we have shown a quantitative defect of both innate and adaptive immune cells in children with DS. In combination with previous literature showing qualitative dysfunction of DS immune cells resulting in decreased chemotaxis, NK-cell activity, and cytotoxicity, [59-65] an important role for the immune system in decreased viral clearance in children with DS is suggested. It is conceivable that loss of immune cells both in quantity and quality will result in higher predisposition to severe respiratory tract infections, such as RSV. Due to a general diminished immune response to respiratory viruses, children with DS are prone to recurrent viral induced wheeze as well, irrespective of RSV.

Conclusion

We have proposed that a disturbed airway physiology and immunology predisposes children with DS to viral respiratory tract infections in general. This hypothesis has been supported by our finding of a high incidence of RSV associated hospitalization and of non-RSV specific recurrent wheeze. In case of children with DS, RSV associated hospitalization would not be the direct cause of recurrent wheeze, but an indication of predisposition to airway pathology that could be equally expressed by recurrent wheeze developed independently from RSV.

Treatment options – the need for a new trial

There is no effective treatment or vaccine available for RSV bronchiolitis. However, since the 90s passive immunization has been shown to decrease the incidence of RSV hospitalization in certain risk groups. [66] In premature born children (less than or equal to 32 weeks gestational age), children with significant CHD or bronchopulmonary dysplasia, passive immunization has been shown to be cost-effective. [67-70] In addition to those strictly defined groups, passive immunization has also been recommended by the world health organization in children with immune deficiency or severe lung pathology such as cystic fibrosis. According to these definitions children with DS would not receive passive immunization unless they had one of these risk factors. Our finding of DS as a new risk factor of RSV associated hospitalization has lead to discussions on the role of passive immunization in this specific group of children. Do we need a new trial or can we assume acceptable tolerability and effectiveness of passive immunization against RSV in children with DS? And if a trial would be needed, would it be feasible to perform such a trial given the relatively low birth rate of children with DS without prematurity and hemodynamically significant heart disease? Would it still be ethical to perform a placebo-controlled trial in this established high-risk population? Although we have shown that children with DS have an increased risk of RSV-associated hospitalization, these children did not have an increased incidence of recurrent wheeze in the long-term

compared to children with DS without a history of RSV-associated hospitalization. This finding has lead to the conclusion that the combination of an aberrant immune system and pulmonary abnormalities result in a higher susceptibility to severe RSV infections in children with DS compared to children without DS based on a possible different mechanism. Consequently, the assumption of acceptable effectiveness of passive immunization against RSV in children with DS is questionable. Passive immunization is an invasive and expensive treatment that has a high burden on patients, their parents and society. In light of ethics, the possibility of unnecessary burden should be taken into account before positive results in certain high-risk populations are generalized to new risk groups. In the Netherlands approximately 300 children with DS are born per year, 50% of which have a second risk factor for severe RSV infections. [44;46] To perform a cost-effective analysis of passive immunization in this specific group, a total of about 1200 children with DS should be included, which would only be feasible in an international multi-centre trial. In conclusion, based on the long-term implications for children with DS and society, an international multi-centre randomized-controlled clinical trial on the use of passive immunization in children with DS is required.

CONCLUSIONS AND FUTURE STUDIES

In this thesis we have shown distinct abnormalities of the innate immune system in children with DS. (*Chapter 3*) A remarkable finding was the increased number of non-classical, pro-inflammatory $CD14^{dim}CD16^{+}$ monocytes in children with DS without any sign of infection at the time of blood collection. It is unclear if the increase in this $CD14^{dim}CD16^{+}$ monocyte subset, as seen in several acute and chronic inflammatory diseases [1-4], is involved in the development of disease or if it expands in response to the inflammatory milieu. The role of increased numbers of $CD14^{dim}CD16^{+}$ monocytes, being either cause or consequence of the high morbidity seen in individuals with DS, needs to be elucidated. In addition to distinct abnormalities of the innate immune system, decreased thymic output was shown to be the cause of decreased number of naive T-cells in children with DS. (*Chapter 4*) In contrast to previous suggestions, thymic insufficiency did not result in a functional defect of naive T-cells as shown by normal or even increased proliferative capacity of these cells in children with DS. The combination of our results and previously reported findings lead to the conclusion that these immunologic abnormalities might result in decreased viral clearance.

The second part of this thesis focused on RSV-associated hospitalization and its sequelae. Down syndrome was shown to be a new independent risk factor of RSV-associated hospitalization. (*Chapter 5*) In contrast to controls, RSV did not add to the risk of recurrent wheeze in children with DS, which was high in children with DS with and

without a history of hospitalization for RSV bronchiolitis. (*Chapter 6*) It was hypothesized that a combination of both immunologic and pulmonary abnormalities leads to increased susceptibility to respiratory tract infections resulting in a high incidence of both RSV-associated hospitalization and virus-induced recurrent wheeze in a parallel way.

The aim of this thesis was to give better insight in the immune system of children with DS and its role in the burden of respiratory tract infections. An attempt was made to translate our findings to use in clinical practice, resulting in the following new clinical and basic immunologic research questions:

1. Is passive immunization against RSV LRTI in children with DS effective, and if so, is health gain achieved at acceptable costs?
2. What is the role of neonatal pulmonary hypertension and lung function abnormalities in the subsequent development of severe RSV infection and recurrent wheeze during the first 2 years of life in children with DS?
3. What is the etiological relationship between a high number of $CD14^{dim}CD16^{+}$ cells during early childhood in children with DS and the subsequent development of infectious and immune-mediated disease?

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Gentianes (2670m)
– Glacier de Tortin (3000m)

Summary

OBJECTIVES

Down syndrome (DS) is the most common chromosomal abnormality in live born children. This thesis started with the clinical observation that children with DS were hospitalized because of respiratory syncytial virus (RSV) more frequently than other children and often with a complicated course of disease. Over the years the immune system of patients with DS has been subject of study to clarify the clinical problems frequently seen in this specific population. The aim of this thesis was to study whether children with DS have an increased risk of severe RSV infections and how this could be mediated by an impaired innate and adaptive immune system.

CHAPTER 1. GENERAL INTRODUCTION

In the Netherlands the incidence of DS is 1 in 650 live born children. DS is associated with gastro-intestinal disease, congenital heart disease, leukemia, several auto-immune diseases and Alzheimer's disease. In addition a high incidence of respiratory morbidity is seen in children with DS. This co-morbidity might be explained by an impaired immune system in patients with DS.

The immune system can roughly be divided in two systems, the innate and adaptive immunity. The innate immune system is important in the first-line defence against microbes, consisting of granulocytes, natural killer (NK) cells, monocytes, invariant natural killer T-cells (iNKTs) and dendritic cells (DCs). All cell types have unique characteristics and function resulting in (in)direct antimicrobial activity.

Naïve T-cells are produced by the thymus, which declines significantly with age. However, relatively constant numbers of naïve T-cells are found in the peripheral blood throughout life, suggesting a certain homeostasis of the human naive T-cell pool. The number of naïve T-cells is maintained by on one side thymic output, peripheral expansion and longevity of existing naïve T-cells and on the other side apoptosis and clonal expansion to a memory or effector phenotype upon antigen stimulation.

RSV is the single-most important cause of lower respiratory tract infections (LRTIs) in infants and young children. Virtually all children are infected with RSV before the age of 2 years and 0.5% to 2% of children require hospitalization. Premature birth, chronic lung disease and congenital heart disease are clinical risk factors for severe disease after RSV infection. The pathophysiology of severe RSV infections is not fully understood, but it is clear that both innate and adaptive host immune responses determine the outcome. Currently no anti-viral treatment or vaccination is available, but passive immunization with monoclonal antibodies reduces the risk of RSV associated hospitalization and has been approved for the previously described risk groups.

Forty to seventy percent of children hospitalized for severe RSV infections develop recurrent wheeze. Although controversies in literature exist, genetic polymorphism studies have shown that early wheeze should be distinguished from late wheeze following RSV, with early wheeze being distinct from allergic asthma.

CHAPTER 2. INCREASED RISK OF RESPIRATORY TRACT INFECTIONS IN CHILDREN WITH DOWN SYNDROME: THE CONSEQUENCE OF AN ALTERED IMMUNE SYSTEM

Respiratory tract infections (RTIs) are the most important cause of mortality in patients with DS at all ages. LRTIs are the main cause of hospitalization in children with DS and when resulting in mechanical ventilation a higher incidence of acute lung injury and acute respiratory distress syndrome has been shown. In children with DS several pathophysiological mechanisms are possibly involved in the development of RTIs: anatomic abnormalities of the respiratory tract, aspiration, hypotonia, and cardiac defects. In recent decades immunologic abnormalities have been elucidated in DS which may contribute to the increased frequency of RTIs in these children as well.

In children with DS decreased numbers and activity of NK-cells have been found. Although conflicting results have been reported, most studies have shown a more or less disturbed function of the innate immune cells resulting in decreased chemotaxis, normal or decreased phagocytosis and increased apoptosis. Studies on DS thymus have provided evidence of accelerated involution of the thymus, an altered pattern of maturation of thymocytes and indications of inefficient thymic output. Decreased numbers of both $CD4^+$ and $CD8^+$ T-cells have been reported in patients with DS, especially naïve T-cells and a relative increase of memory T-cells. Several functional assays have revealed decreased proliferation and cytotoxicity of T-cells in patients with DS. Besides T-cells, B-cell counts were found to be lower in children with DS as well. Several studies on immunoglobulin levels in patients with DS have been performed with conflicting results. Although IgM antibodies production was shown to be normal or decreased in DS, levels of other antibodies were normal or even increased (IgA, IgG), with IgG1 and IgG3 usually increased and IgG2 and IgG4 decreased. An increased loss of telomeric length in subpopulations of T-cells, B-cells and neutrophils has been shown in children with DS. Children with DS showed similar tendency to undergo apoptosis compared with controls, both unstimulated and after stimulation with apoptogenic drugs.

In conclusion, patients with DS show multiple abnormalities both in numbers and function of both innate and adaptive immunity. These abnormalities combined, whether or not directly interacting with each other, strongly suggest diminished viral and bacterial clearance in DS. Although it can be suspected that certain findings reflect

a state of inflammation in DS rather than being the cause, it was hypothesized that the high incidence of respiratory tract infections in children with DS is the consequence of an impaired immune system.

CHAPTER 3. DISTINCT ABNORMALITIES IN THE INNATE IMMUNE SYSTEM OF CHILDREN WITH DOWN SYNDROME

In this chapter we studied the hypothesis that children with DS have abnormal number of innate immune cells in the peripheral blood. Children with DS showed significantly lower absolute total leukocyte counts, lymphocytes, monocytes and granulocytes. However, absolute numbers of $CD14^{dim}CD16^{+}$ monocyte were 1.5 times higher in DS compared to controls. This was fully explained by a higher percentage of $CD14^{dim}CD16^{+}$ monocytes within the monocyte compartment. Consequently, absolute counts and percentages of the $CD14^{+}CD16^{-}$ monocyte population were significantly decreased in children with DS, which was independent from sex and age. Children with DS showed significantly lower absolute counts of mDCs. Numbers of pDCs and NK cell counts were normal. Absolute numbers of invariant NKT cells were very low overall, but significantly lower in children with DS than in controls. In conclusion, distinct abnormalities of cells of the innate immune system were shown in children with DS. The high frequency of $CD14^{dim}CD16^{+}$ monocytes may play an important role in the onset and maintenance of chronic inflammatory disease in children with DS.

CHAPTER 4. DECREASED THYMIC OUTPUT ACCOUNTS FOR DECREASED NAIVE T-CELL NUMBERS IN CHILDREN WITH DOWN SYNDROME

In order to improve our understanding of low naïve T-cells in children with DS we studied T-cell dynamics. The relative contribution of thymic insufficiency, peripheral generation and loss by antigen driven differentiation or apoptosis, to decreased numbers of naïve T-cells in children with DS was compared to age-matched controls. The total number of T-cell receptor excision circles (TREC) per ml blood was more than two fold decreased in children with DS compared to controls. Reduced frequencies and absolute numbers of protein tyrosine kinase 7 positive ($PTK7^{+}$) recent thymic emigrants were found in children with DS. No differences in the level of peripheral naive T-cell loss between children with DS and healthy controls were shown. The fraction of naive T-cells expressing the proliferation marker Ki67 or CD31 was similar compared to healthy controls and therefore do not support a defective peripheral generation of naive T-cells in children with DS. Although significantly higher IL-7 plasma levels were found in children with DS,

a normal capacity to respond to IL-7 was shown. No significant changes were found in the TREC content of naive $CD4^+$ T-cells in children with DS. For naive $CD8^+$ T-cells not an increased but even a decreased TREC content was found, pointing to increased survival or peripheral generation of naive T-cells in DS. We hypothesized that these mechanisms might counteract reduction of thymic output but consequently would lead to loss of diversity of the T-cell receptor repertoire, which was shown for a small subgroup of children with DS compared to controls. The combined results of our in detail study of T-cell dynamics now put forward that low naïve T-cell numbers in children with DS can be fully explained by insufficient thymic output, but not reduced peripheral generation nor increased loss of naive T-cells.

CHAPTER 5. DOWN SYNDROME: A NOVEL RISK FACTOR FOR RESPIRATORY SYNCYTIAL VIRUS BRONCHIOLITIS— A PROSPECTIVE BIRTH-COHORT STUDY

Although respiratory morbidity is a common problem of children with DS addressed in daily clinical practice, only a few reports have described the extent of this issue. A retrospective observational study was combined with a prospective national birth cohort of children with DS to estimate the true incidence of RSV-associated hospitalization. Of the 395 patients with DS, 180 (45.6%) had a known risk factor for severe RSV infections; 39 (9.9%) of these were hospitalized for RSV LRTI. Two out of 276 control children (0.7%) were hospitalized for RSV LRTI. The median duration of hospitalization was 10 days; 31 children (79.5%) required supplemental oxygen and mechanical ventilation was required for five children (12.8%). The OR for RSV LRTI-associated hospitalization was 12.6 (95% confidence interval: 2.9-54.5) among term children with DS without hemodynamically significant CHD and 10.5 (95% confidence interval: 2.2-49.5) among term children with DS without any CHD. It was thus concluded that DS is a novel independent risk factor for severe RSV LRTI.

CHAPTER 6. HIGH INCIDENCE OF RECURRENT WHEEZE IN CHILDREN WITH DOWN SYNDROME WITH AND WITHOUT PREVIOUS RESPIRATORY SYNCYTIAL VIRUS LOWER RESPIRATORY TRACT INFECTION

A combined retrospective cohort and prospective birth cohort of children with DS with a history of hospitalization for RSV-induced LRTI was studied (n=53). Three control populations were included: children with DS without hospitalization for RSV-induced LRTI (n=110), children without DS but with hospitalization for RSV-induced LRTI (n=48), and healthy siblings of the previous three groups mentioned (n=49). The primary outcome

was the incidence of physician-diagnosed recurrent wheeze up to two years of age. This study showed that RSV-induced LRTI does not have a significant effect on the incidence of recurrent wheeze in children with DS (DS+ RSV+ 36% versus DS+ RSV- 30%). In children without DS physician-diagnosed wheeze was found more frequently in children hospitalized for RSV-induced LRTI (31%) than healthy controls (8%). Logistic regression analysis confirmed that hospitalization for RSV-induced LRTI altered the risk of recurrent wheeze only in children without DS, but not in children with DS. An unexpected finding was that recurrent wheeze was very common among children with DS.

CHAPTER 7. GENERAL DISCUSSION

Whether the distinct abnormalities we have described in the innate immunity of children with DS are cause or consequence is unclear. Although children with DS did not show any signs of infection at time of blood collection, high numbers of $CD14^{dim}CD16^{+}$ monocytes might be the consequence of subclinical chronic inflammation. Alternatively, increased numbers of $CD14^{dim}CD16^{+}$ monocytes may be the actual cause of the high morbidity, playing a role in the susceptibility to auto-immune diseases in individuals with DS later in life.

Previous literature on immunology in patients with DS has suggested that abnormal thymic development and function might cause the T-cell abnormalities shown in children with DS. Our study on the dynamics of the naïve T-cell compartment has confirmed that abnormal thymic development causes low number of naïve T-cells in children with DS, and expanded our knowledge by showing that peripheral mechanisms regulating the size of the naïve T-cell pool appear intact.

A model of parallel predisposition to viral RTIs has been proposed to explain the high incidence of both RSV-infections and recurrent wheeze in children with DS. Both a disturbed airway physiology and a quantitative or qualitative loss of immune cells might result in a higher susceptibility to severe RTIs. In case of children with DS, RSV-associated hospitalization would not be the direct cause of recurrent wheeze, but an indication of predisposition to airway pathology that could be equally expressed by recurrent wheeze developed independently from RSV.

Our finding of DS as a new risk factor of RSV associated hospitalization has lead to discussions on the role of passive immunization in this specific group of children. The need, ethics and feasibility of a new trial are discussed leading to the conclusion that based on the implications for children with DS and society, a randomized-controlled clinical trial on the use of passive immunization in children with DS is required.

Other future studies that are suggested include a birth cohort study of lung function and pulmonary hypertension related to clinical data in children with DS to examine the

role of airway physiology in the predisposition to RTIs and recurrent wheeze. Finally, an immunologic mouse model of DS will give the opportunity to study the development of the immune system and the influence of acute and chronic infections on the distribution of different cell subsets. This in order to determine whether the immunologic abnormalities found in individuals with DS are the cause or consequence of the high incidence of (auto-immune) diseases.


4

Nederlandse Samenvatting

Col des Gentianes (2950m) – Mont-Fort (3330m)

DOELSTELLING

Down syndroom (DS) is de meest voorkomende chromosomale afwijking bij (levende) pasgeborenen. Aan de basis van dit proefschrift lag de klinische observatie dat kinderen met DS vaker dan andere kinderen werden opgenomen in het ziekenhuis in verband met respiratoir syncytieel virus (RSV[1]). Bovendien leken zij een ernstiger ziektebeloop te hebben dan kinderen zonder DS. De afgelopen 30 jaar zijn er verschillende studies naar het immuun systeem van patiënten met DS verschenen in de hoop de klinische problemen die frequent gezien worden in deze specifieke groep te kunnen verklaren. Het doel van dit proefschrift was om te bestuderen of kinderen met DS een verhoogd risico op ernstige RSV infecties hebben (zoals de klinische observatie deed vermoeden) en vervolgens hoe stoornissen van het afweersysteem hier een rol bij kunnen spelen.

HOOFDSTUK 1. ALGEMENE INLEIDING

De incidentie van DS in Nederland is ongeveer 1 op 650 levende pasgeboren kinderen. Er bestaat een duidelijke associatie tussen DS en maag-darm aandoeningen, aangeboren hartafwijkingen, leukemie, verschillende auto-immuunziekten en de ziekte van Alzheimer. Bovendien wordt een hoge incidentie van luchtweg aandoeningen bij kinderen met DS gezien. Deze co-morbiditeit zou verklaard kunnen worden door een afwijkend afweersysteem bij patiënten met DS. Het afweersysteem kan ruwweg in twee verschillende systemen worden onderverdeeld: het innate immuun systeem en het adaptieve immuun systeem. Het innate immuun systeem is het aangeboren, onrijpe afweersysteem dat het eerst in actie komt als een ziekteverwekker het lichaam binnen dringt. Granulocyten, natural killer (NK) cellen, monocyten, invariante natural killer T-cellen (iNKTs) en dendritische cellen (DCs) behoren tot het innate immuun systeem. Elk celtype heeft unieke kenmerken en functies die zorgen voor (in)directe antimicrobiële activiteit.

Het adaptieve immuun systeem wordt ook wel het specifieke afweersysteem genoemd en wordt slechts actief als het innate immuun systeem specifiek herkenbare deeltjes presenteert aan het adaptieve immuunsysteem. Het adaptieve immuunsysteem bestaat voornamelijk uit T- en B-cellen. Binnen deze celtypes wordt onderscheid gemaakt allereerst tussen $CD4^+$ en $CD8^+$ T-cellen en vervolgens tussen naïeve cellen, memory cellen en effector cellen. $CD4^+$ T-cellen worden ook wel helper cellen genoemd omdat zij een aansturende functie hebben. $CD8^+$ T-cellen worden ook wel cytotoxische T-cellen genoemd omdat zij met name een rol hebben in het doden van geïnfecteerde

1 RSV is een van meest voorkomende veroorzakers van luchtweginfecties bij kinderen.

cellen. Naïeve T-cellen worden door de thymus[2] geproduceerd en worden naïef genoemd omdat ze nog niet in aanraking zijn geweest met antigenen[3]. De productie door de thymus wordt significant minder naarmate je ouder wordt. Desondanks blijft het aantal naïeve T-cellen in het bloed relatief constant gedurende je hele leven. Dit suggereert het bestaan van een homeostatisch mechanisme van het humane naïeve T-cel systeem. Het aantal naïeve T-cellen kan in evenwicht gehouden worden door enerzijds nieuwe productie van naïeve T-cellen door de thymus (thymus output), perifere expansie en vergrote levensduur van de cellen en anderzijds door celdood (apoptosis) en clonale expansie naar een memory of effector fenotype[4] als gevolg van antigene stimulatie.

RSV is de belangrijkste oorzaak van lagere luchtweginfecties (LLWI) bij baby's en peuters. Bijna alle kinderen maken een primaire RSV-infectie door voor de leeftijd van 2 jaar. De meeste kinderen ontwikkelen slechts symptomen van een bovenste luchtweginfectie (LWI), maar 1-2% van hen wordt zieker met symptomen van een onderste luchtweginfectie, waarvoor ze opgenomen moeten worden in het ziekenhuis in verband met voedingsproblemen of zuurstofbehoefte. Bij ongeveer 10% van deze kinderen zijn de symptomen zo ernstig dat zij op de intensive care voor kinderen worden opgenomen. Prematuriteit, chronische longziekte en aangeboren hartafwijkingen zijn bekende klinische risicofactoren voor een ernstig ziektebeloop als gevolg van een RSV infectie. De pathofysiologie[5] van ernstige RSV infecties is niet volledig begrepen. Wel is duidelijk dat de reactie van zowel het innate als het adaptieve immuun systeem het ziektebeloop bepalen. Tot op heden is er geen vaccin of behandeling met anti-virale middelen beschikbaar. Passieve immunisatie[6] verlaagt echter het risico op hospitalisatie geassocieerd met RSV. De toepassing van passieve immunisatie wordt vergoed in de eerder genoemde risicogroepen.

Veertig tot zeventig procent van de kinderen die in het ziekenhuis zijn opgenomen in verband met een ernstige RSV infectie, ontwikkelt periodieke klachten van een piepende ademhaling (wheeze). Hoewel er veel discussie in de literatuur bestaat, hebben genetische studies aangetoond dat er een onderscheid gemaakt moet worden tussen het vroeg en laat ontstaan van wheeze klachten na het doormaken van een ernstige RSV infectie. In het bijzonder, de wheeze klachten die vroeg na de RSV infectie zijn ontstaan, zijn duidelijk verschillend van astma.

2 Thymus = zwezerik

3 Antigenen zijn moleculen die het afweersysteem aanzetten tot het maken van o.a. antistoffen. Een voorbeeld van een antigen is een virus of bacterie, waarvoor dan ook wel de term pathogeen wordt gebruikt aangezien dit kan leiden tot ziekte (Grieks *pathos*) .

4 Fenotype is het totaal aan waarneembare kenmerken van een organisme, ook wel verschijningsvorm.

5 Pathofysiologie is de ontstaanswijze van een ziekte, ook wel ziektemechanisme.

6 Passieve immunisatie is het direct inspuiten van antistoffen (afweerstoffen) tegen een bekende verwekker om zo de persoon te beschermen tegen de gevolgen van de infectie.

HOOFDSTUK 2. EEN VERHOOGD RISICO OP LUCHTWEGINFECTIES BIJ KINDEREN MET DOWN SYNDROOM: DE CONSEQUENTIE VAN EEN VERANDERD IMMUUN SYSTEEM

Luchtweginfecties (LWIs) zijn de belangrijkste oorzaak van mortaliteit bij kinderen met DS op alle leeftijden. LWIs zijn de belangrijkste oorzaak van hospitalisatie bij kinderen met DS en indien deze kinderen beademd moeten worden leidt dit vaker tot longschade. Verschillende pathofysiologische mechanismen bij kinderen met DS kunnen bijdragen aan het ontstaan van LWIs: anatomische afwijkingen van de luchtwegen, verslikking (aspiratie), verlaagde spierspanning (hypotonie) en hartafwijkingen. In de afgelopen decennia zijn tevens afwijkingen van het afweersysteem van kinderen met DS gevonden, die een rol zouden kunnen spelen bij de verhoogde gevoeligheid voor LWIs.

Bij kinderen met DS is een verlaagd aantal en verminderde activiteit van NK-cellen gevonden. Hoewel tegenstrijdige resultaten zijn beschreven, laten de meeste studies in meer of mindere mate afwijkingen van de functie van het innate immuun system zien: verminderde chemotaxis[7], normale of verminderde fagocytose[8] en versterkte apoptosis[9]. Studies naar de thymus bij DS hebben aangetoond dat de thymus versneld involueert, dat de thymus cellen (thymocyten) een ander rijpingspatroon hebben en indicaties voor een inefficiënte thymus output. Kinderen met DS hebben een verminderd aantal $CD4^+$ en $CD8^+$ T-cellen, in het bijzonder naïeve T-cellen Het aantal memory T-cellen echter, is relatief verhoogd. Verschillende functionele studies hebben een verminderde proliferatie[10] en cytotoxiciteit[11] van T-cellen bij kinderen met DS laten zien. Naast T-cellen zijn B-cellen ook verlaagd bij kinderen met DS. Verschillende studies over immunoglobulines[12] bij kinderen met DS leveren tegenstrijdige resultaten op. Enerzijds wordt een verlaagde of normale productie van IgM antilichamen bij DS gezien. Anderzijds zijn de waarden van andere antilichamen normaal of zelfs verhoogd (IgA, IgG), waarbij IgG1 en IgG3 meestal verhoogd terwijl IgG2 en IgG4 juist verlaagd zijn. Een sterker verlies aan lengte van telomeren[13] binnen subpopulaties van T-cellen, B-cellen en neutrofielen is gevonden bij kinderen met DS vergeleken met gezonde controles. De mate van apoptosis is

7 Chemotaxis is het verschijnsel dat organismen zich verplaatsen als gevolg van verschillen in concentratie van bepaalde stoffen in de omgeving.

8 Fagocytose is het proces waarbij een cel een andere cel omsluit om deze vervolgens af te breken.

9 Apoptosis is geprogrammeerde celdood, waarbij een cel als gevolg van bijvoorbeeld celschade of infectie wordt aangezet om zichzelf te doden.

10 Proliferatie = celdeling

11 Cytotoxiciteit is het vermogen om een andere cel te doden.

12 Immunoglobulines (Ig) zijn antistoffen die door de B-cel worden geproduceerd. Onder verschillende omstandigheden worden verschillende subgroepen gemaakt: IgM, IgG, IgA, IgE, IgD.

13 Telomeren zijn de uiteindes van chromosomen, waarbij tijdens elke celdeling een stukje wordt verwijderd. Na ongeveer 50-60 delingen is het uiteinde zo kort geworden dat er geen celdeling meer kan optreden en de cel sterft.

vergelijkbaar bij kinderen met en zonder DS, zowel ongestimuleerd als na stimulatie met apoptosis bevorderende middelen.

Concluderend hebben kinderen met DS verschillende afwijkingen zowel in aantal als in functie van het innate en het adaptieve immuun systeem. De combinatie van deze afwijkingen (of er nu een directe communicatie over en weer is of niet) suggereert dat het vermogen om virussen en bacteriën op te ruimen, verminderd is. Hoewel bepaalde bevindingen een weerspiegeling zouden kunnen zijn van een inflammatoire[14] toestand in plaats van een causaal gevolg, is onze hypothese dat een hoge incidentie van LWIs bij kinderen met DS de consequentie is van een verstoord immuun systeem.

HOOFDSTUK 3. STERK VERSCHILLENDE AFWIJKINGEN IN HET INNATE IMMUUN SYSTEEM BIJ KINDEREN MET DOWN SYNDROOM

In dit hoofdstuk hebben wij de hypothese bestudeerd dat kinderen met DS een abnormaal aantal innate immuun cellen in hun bloed hebben vergeleken met gezonde controles. Kinderen met DS bleken een significant lager absoluut aantal leukocyten[15], lymphocyten, monocyten en granulocyten te hebben. Opvallend was de bevinding dat het absolute aantal $CD14^{dim}CD16^{+}$ monocyten[16] 1.5 keer zo hoog was bij kinderen met DS in vergelijking tot controles. Dit werd volledig verklaard door een hoger percentage $CD14^{dim}CD16^{+}$ monocyten binnen het monocyten compartiment. Daarom waren het absolute aantal en de percentages van de $CD14^{+}CD16^{-}$ monocyten significant verlaagd bij kinderen met DS. Dit effect was onafhankelijk van geslacht en leeftijd. Kinderen met DS hadden een significant lager absoluut aantal mDCs[17]. Het aantal pDCs en NK-cellen was normaal. Absolute aantallen iNKT cellen waren in beide groepen erg laag, maar significant lager bij kinderen met DS ten opzichte van kinderen zonder DS. Concluderend werd een sterk afwijkend aantal innate immuun cellen getoond bij kinderen met DS. Het verhoogde aantal $CD14^{dim}CD16^{+}$ monocyten zou een belangrijke rol kunnen spelen in het ontwikkelen en de instandhouding van chronische inflammatoire ziekten bij kinderen met DS.

14 Inflammatie = ontsteking

15 Leukocyt betekent "witte bloedcel", hiertoe behoren vrijwel alle cellen van het afweersysteem

16 Monocyten kunnen weer verder worden onderverdeeld in klassieke $CD14^{+}CD16^{-}$ en de niet-klassieke, pro-inflammatoire $CD14^{dim}CD16^{+}$ monocyten.

17 Binnen dendritische cellen (DCs) wordt onderscheid gemaakt tussen myeloid DCs (mDCs) en plasmacytoid DCs (pDCs), elk met hun eigen specifieke rol in het innate immuun systeem.

HOOFDSTUK 4. VERMINDERDE THYMUS OUTPUT IS DE OORZAAK VAN HET VERMINDERD AANTAL NAÏEVE T-CELLEN BIJ KINDEREN MET DOWN SYNDROOM

Ten einde de oorzaak van het verlaagd aantal naïeve T-cellen bij kinderen met DS te achterhalen hebben wij de T-cel dynamica bestudeerd. De relatieve bijdrage van thymus insufficiëntie, perifere aanmaak en verlies (als gevolg van antigeengestimuleerde differentiatie[18] of apoptosis) aan het verlaagd aantal naïeve T-cellen bij kinderen met DS werd vergeleken met kinderen zonder DS van overeenkomstige leeftijd. Het totaal aantal T-cel receptor excisie cirkels (TRECs)[19] per ml bloed was meer dan twee keer verlaagd bij kinderen met DS in vergelijking met controles. Verlaagde percentages en absolute aantallen van proteïne tyrosine kinase 7 positieve ($PTK7^+$)[20] T-cellen werden gevonden bij kinderen met DS. Er werd geen verschil gevonden in het verlies van perifere naïeve T-cellen tussen kinderen met en zonder DS. Het percentage naïeve T-cellen dat de proliferatie marker Ki67 of CD31 tot expressie brengt op het celoppervlak was vergelijkbaar met gezonde controles. Dit suggereert dat de perifere aanmaak van naïeve T-cellen intact is bij kinderen met DS. Hoewel een significant hogere waarde van IL-7[21] in het bloed werd gevonden bij kinderen met DS, werd een normaal vermogen van de naïeve T-cel om te reageren op IL-7 aangetoond. Er werd geen verschil in de TREC inhoud van naïeve $CD4^+$ T-cellen bij kinderen met DS gevonden. De TREC inhoud van naïeve $CD8^+$ T-cellen was zelfs verlaagd, wat wijst op verhoogde levensduur of perifere aanmaak van naïeve T-cellen bij kinderen met DS. De hypothese die hieruit voort komt is dat deze mechanismen de afname in thymus output compenseren. De consequentie van deze compensatie is echter dat dit zou kunnen leiden tot verlies in het T-cel receptor repertoire, wat werd bevestigd in een kleine subgroep van patiënten in vergelijking met controles. Alle resultaten van deze gedetailleerde studie over T-cel dynamica gecombineerd brengt ons tot de conclusie dat een laag aantal naïeve T-cellen bij kinderen met DS volledig verklaard kan worden door insufficiënte thymus output, maar niet door verlaagde perifere aanmaak of een verhoogd verlies van naïeve T-cellen.

18 Differentiatie is het proces waarbij een cel zich ontwikkelt tot een cel met verschillende eigenschappen, bijvoorbeeld van een cel met naïeve kenmerken tot een cel met eigenschappen van een memory cel.

19 Een TREC is een cirkel van DNA die aanwezig is in een T-cel als hij net door de thymus is geproduceerd. Tijdens een celdeling wordt deze cirkel slechts aan één van de twee dochtercellen doorgegeven. Bij elke celdeling wordt het aantal T-cellen met een TREC in zich verdund, dus hoe meer celdelingen plaats vinden hoe lager het aantal TREC bevattende T-cellen wordt.

20 PTK7 is een recent beschreven molecuul dat op het oppervlak voorkomt van T-cellen die onlangs door de thymus geproduceerd zijn en daarmee geduid worden als de meest naïeve T-cellen die in het bloed aanwezig zijn.

21 IL-7 is een eiwit dat kan binden aan naïeve T-cellen om deze tot proliferatie aan te zetten zonder dat de T-cel daarbij zijn naïviteit verliest. Een hoge waarde van dit eiwit in het bloed kan duiden op onvoldoende capaciteit van de T-cel om IL-7 te binden.

HOOFDSTUK 5. DOWN SYNDROOM: EEN NIEUWE RISICOFACTOR VOOR RESPIRATOIR SYNCYTIEEL VIRUS BRONCHIOLITIS—EEN PROSPECTIEVE GEBOORTE COHORT STUDIE

Hoewel luchtwegklachten een veel voorkomend probleem zijn bij kinderen met DS in de dagelijkse klinische praktijk, zijn er slechts enkele studies die het belang van dit probleem beschrijven in de literatuur. Een retrospectieve, observationele studie werd door ons gecombineerd met een prospectieve studie van een nationaal geboorte cohort van kinderen met DS om de werkelijke incidentie van hospitalisatie als gevolg van RSV vast te stellen. Van de 395 patiënten met DS hadden 180 kinderen (45.6%) een bekende risicofactor voor ernstige RSV-infecties; 39 (9.9%) van hen zijn daadwerkelijk gehospitaliseerd vanwege een RSV LLWI. Twee van de 276 controle kinderen zonder DS (0.7%) werden eveneens gehospitaliseerd vanwege een RSV LLWI. De mediaan van de opnameduur was 10 dagen; 31 kinderen (79.5%) hadden extra zuurstof behoefte en 5 kinderen hadden beademing nodig (12.8%). De Odds ratio (OR) voor hospitalisatie geassocieerd met RSV LLWI was 12.6 (95% confidentie interval: 2.9-54.5) bij a terme[22] geboren kinderen met DS zonder hemodynamisch significante congenitale hartafwijking (CHA)[23] en 10.5 (95% confidentie interval: 2.2-49.5) voor a terme geboren kinderen met DS zonder enige vorm van CHA. Concluderend is DS een nieuwe, onafhankelijke risicofactor voor ernstige RSV LLWIs.

HOOFDSTUK 6. HOGE INCIDENTIE VAN PERIODIEKE WHEEZE KLACHTEN BIJ KINDEREN MET DOWN SYNDROOM MET EN ZONDER EEN VOORGESCHIEDENIS VAN RESPIRATOIR SYNCYTIEEL VIRUS LAGERE LUCHTWEGINFECTIE

Een gecombineerd retrospectieve cohort en een prospectief geboortecohort van kinderen met DS met in de voorgeschiedenis hospitalisatie in verband met een RSV LLWI werd bestudeerd (n=53). Drie controle populaties werden geïncludeerd: kinderen met DS zonder hospitalisatie in verband met RSV LLWI (n=110), kinderen zonder DS maar met hospitalisatie in verband met RSV LLWI (n=48), en gezonde broertjes en zusjes van de eerste drie groepen (n=49). De primaire uitkomstmaat was de incidentie van periodieke wheeze klachten door een arts gediagnosticeerd in de eerste 2 jaar. De resultaten van deze studie laten zien dat een RSV LLWI geen significant effect heeft op de incidentie

22 A terme betekent geboren bij een normale zwangerschapsduur van 40 weken in tegenstelling tot prematuriteit waarbij een kind onder de 37 weken zwangerschapsduur geboren wordt

23 Een hemodynamisch significante hartafwijking betekent een hartafwijking die een duidelijk negatief effect heeft op de mate van bloedsomloop en daarmee de functie van het hart

van periodieke wheeze klachten bij kinderen met DS (DS+ RSV+ 36% versus DS+ RSV- 30%). Bij kinderen zonder DS werden vaker periodieke wheeze klachten gevonden in de groep met hospitalisatie in verband met RSV LLWIs (31%) dan in de gezonde broertjes en zusjes (8%). Logistische regressie analyse bevestigde dat hospitalisatie vanwege een RSV LLWI het risico op periodieke wheeze klachten alleen bij kinderen zonder DS verhoogde, maar niet bij kinderen met DS. Een onverwachte bevinding was dat periodieke wheeze klachten zeer frequent voorkomen bij kinderen met DS.

HOOFDSTUK 7. ALGEMENE DISCUSSIE

Het is onduidelijk of de belangrijke verschillen die wij vonden in het innate immuun systeem van kinderen met DS oorzaak of gevolg zijn van de ziekten die in deze specifieke populatie gezien worden. Hoewel de kinderen met DS uit onze studie geen tekenen van infectie hadden ten tijde van de bloedafname, zou het verhoogde aantal $CD14^{dim}CD16^{+}$ monocyten de consequentie kunnen zijn van subklinische chronische inflammatie. Anderzijds zou dit verhoogde aantal $CD14^{dim}CD16^{+}$ monocyten juist de oorzaak kunnen zijn van de hoge morbiditeit[24], waarbij dit een rol speelt in de gevoeligheid voor auto-immuun ziekten[25] op latere leeftijd bij mensen met DS.

Eerdere literatuur over immunologie bij patiënten met DS heeft gesuggereerd dat een abnormale ontwikkeling en functie van de thymus, de T-cel afwijkingen bij kinderen met DS zou veroorzaken. Onze studie naar naïeve T-cel dynamica heeft bevestigd dat abnormale thymus ontwikkeling een verlaagd aantal naïeve T-cellen tot gevolg heeft bij kinderen met DS. Daarbij is onze kennis verder toegenomen doordat wij aangetoond hebben dat perifere mechanismen, die eveneens invloed hebben op de grootte van het naïeve T-cel compartiment, intact lijken te zijn bij kinderen met DS.

In dit hoofdstuk stellen wij een model voor van een parallelle predispositie voor virale LWIs om zo de hoge incidentie van zowel RSV LLWIs en periodieke wheeze klachten te kunnen verklaren bij kinderen met DS. Zowel een afwijkende anatomie en werking van de luchtwegen als een kwantitatief of kwalitatief verlies van immuun cellen zou kunnen resulteren in een hogere gevoeligheid voor en ernstiger beloop van LWIs. In het geval van kinderen met DS zou hospitalisatie in verband met een RSV LLWI niet de directe oorzaak van periodieke wheeze klachten zijn, maar een indicatie van predispositie voor luchtweg pathologie dat evenzeer wordt aangetoond door periodieke wheeze klachten die onafhankelijk van RSV ontstaan.

24 Morbiditeit = ziektecijfer.

25 Auto-immuun ziekten zijn aandoeningen waarbij het afweersysteem lichaamseigen cellen aanziet als lichaamsvreemd, waarbij dan antistoffen tegen de eigen weefsels worden gevormd.

Onze bevinding van DS als een nieuwe risicofactor voor hospitalisatie als gevolg van RSV LLWIs heeft geleid tot discussies over de rol van passieve immunisatie in deze specifieke groep kinderen. De noodzaak, ethiek en haalbaarheid van een nieuwe studie worden in dit hoofdstuk besproken. Dit leidt tot de conclusie dat op basis van de implicaties voor kinderen met DS en de maatschappij, een gerandomiseerde, placebo-gecontroleerde klinische studie naar het gebruik van passieve immunisatie bij kinderen met DS nodig is.

Andere suggesties voor toekomstige studies bevatten een geboorte cohort studie naar longfunctie en pulmonale hypertensie in relatie tot klinische uitkomst bij kinderen met DS. Zo kan de rol van de luchtwegen van kinderen met DS bij de predispositie voor LWIs en periodieke wheeze klachten worden onderzocht. Tot slot geeft een immunologisch muismodel van DS[26] de kans om de ontwikkeling van het immuun systeem te onderzoeken en de invloed van acute en chronische infecties op de verdeling van verschillende immuun cellen te bepalen. Op deze manier kan achterhaald worden of de immunologische afwijkingen bij individuen met DS de oorzaak of het gevolg zijn van de hoge incidentie van (auto-immuun) ziekten.

26 Veel medisch wetenschappelijk onderzoek maakt gebruik van proefdiermodellen waarbij een ziekte, zoals die gezien wordt bij de mens, wordt nagebootst bij een proefdier, bijvoorbeeld de muis. Zo is dan ook een muismodel ontwikkeld van Down syndroom, waarbij de muis een extra chromosoom (in dit geval 16 ipv 21) heeft. Daarmee lijkt deze muis in veel opzichten op een patiënt met Down syndroom. Hierdoor kunnen bepaalde aspecten, zoals bijvoorbeeld het immuunsysteem, onderzocht worden zonder hiervoor patiënten te belasten.

Croix de Coeur (2174m)
– Petit Combin (3672m)

Dankwoord

DANKWOORD

Slechts enkele stappen verwijderd van de top, waarvandaan ik straks met een voldaan gevoel kan terug kijken op de afgelegde route, lijkt het een goed moment om stil te staan bij de mensen die me in deze tocht hebben geholpen. Want ook al maakt de techniek het je tegenwoordig gemakkelijk door je in een paar minuten honderden meters omhoog te hijsen, voor bepaalde toppen moet je het toch nog steeds op eigen kracht doen en dat lukt je alleen met de hartverwarmende ondersteuning van de vele patientjes, collega's, vrienden en familie die me in voor- en tegenspoed met raad, daad, koffie en thee hebben bijgestaan. Het is dan ook aan hen aan wie ik dit hoofdstuk opdraag.

Zonder wetenschap geen zorg, zonder zorg geen wetenschap
Allereerst wil ik alle kinderen met Down syndroom en hun ouders bedanken voor hun medewerking aan dit onderzoek. De belangrijkste drive om via diepe dalen hoge toppen te beklimmen is de impact die je hoopt te bewerkstelligen als je het einddoel weet te bereiken. De kus op je wang van een kind met Down syndroom na een eerste boze blik als je ze hebt geprikt, is minstens zoveel waard als het bereiken van de top.

The real leader has no need to lead - he is content to point the way (H. Miller)
Jan, de getrainde afstandsloper met een lange adem. Jij hebt mij het startsein gegeven en met concrete, op ervaring gebaseerde adviezen vanaf de zijlijn me in alle rust naar de finish geleid.

Marceline, de fietsster binnen deze expeditie. In de juiste versnelling, met een frisse wind en dankzij je kritische blik hebben we vele etappes van deze tocht zeer succesvol weten te finishen.

Louis, de allrounder in het team. Organisatorisch of inhoudelijk, klinisch of basaal immunologisch, werk of privé, je bent van alle markten thuis. Onze werkbesprekingen hadden het effect van een pakje dextro: een boost aan energie en zeer verslavend!

The knowledge of the world is only to be acquired in the world, and not in a closet (Lord P. Chesterfield)
Behalve deze kerngroep, kom je nog heel wat mensen tegen in die jaren, die ieder op hun eigen manier hebben bijgedragen aan mijn voortgang naar de top.

Allereerst de RSV-groep met promovendi Marieke, Michiel, Annemieke, Mirjam en Maarten, maar ook alle analisten, researchverpleegkundigen en studenten, waar op een

veilige manier elke week je stappen voor of achteruit geanalyseerd konden worden. Dat groepsgevoel is een onvergetelijke stimulans.

De mensen van het lab, eerst op de 3e en vervolgens op de 2e verdieping, maar ook in de VU, hebben mij als simpele wandelaar uitgerust met voldoende kennis en technieken zodat ik me nu bijna een professionele bergbeklimmer voel. In het bijzonder Leontine en later Sigrid, veel dank voor de praktische begeleiding. Marein en Theo dank voor jullie geduld en toewijding voor het DC-werk, misschien dat het ooit nog wel weer van de plank wordt gehaald…

De andere coauteurs van de publicaties (Chantal, Michel, Reinoud, Jan, Kim, Grada, Kiki, Jose en Roel): vaak konden we alleen op afstand samenwerken, maar de gezamenlijke publicaties mogen er zijn en daarmee hebben we toch fantastische resultaten bereikt!

Na een wat eenzame start, werd ik al snel vergezeld door mijn lieve kamergenootjes. Eerst Anne en later Lieke en Mieke, jullie wisten door kletspraat over thuis en allerhande tips en tricks voor werk en privé heel wat blaren te doen vergeten. Na een vrijdagmiddag thee- (of water-) sessie met koekjes, chocolade of drop kon ik er altijd weer goed gehumeurd en vol energie (letterlijk) tegenaan.

Alle andere collega's uit het WKZ, die ook af en toe een pauze moesten inlassen, hebben ervoor gezorgd dat ik na 5 minuten alweer lichtvoetig door kon stappen. Alma, Martijn en Hubert onze hardlooprondjes waren een perfecte training en heerlijk om alle stress kwijt te raken!

Het afgelopen jaar in het OLVG was druk maar zeer succesvol. Heerlijk om me weer in de kliniek te kunnen inzetten, maar tegelijkertijd tijd over te houden voor de afronding van dit boekje. Lieve collega's, veel dank voor de fijne werkomgeving en de ruimte die jullie me hiervoor hebben gegeven!

Je voelt je thuis daar waar je wordt begrepen (C. Morgenstern)
Ook buiten het werk had ik genoeg mensen die deelden in mijn Up- en Down-gevoel. Familie (Tantien, Babbel en Willemijn) en vrienden (Margot, Sophie, Joyce, Meta, Irene en Jeske) van kleins af aan of sinds de studententijd staan jullie me bij in verdrietige en vreugdevolle tijden. Al gooien onze agenda's regelmatig roet in het eten, gelukkig kunnen we altijd wel een momentje vinden om gewoon te genieten van het leven!

Lieve Marianne en Robert, Flos en Eva, jullie hebben me met heel veel warmte in jullie familie opgenomen. Het doet me veel plezier om te zien wat voor een onuitputtelijke

interesse jullie hebben in mijn onderzoek, ondanks dat het redelijk wat van onze family-time afhaalt. Binnenkort is er hopelijk weer wat meer tijd om gezellige dingen met jullie te doen. Welke bergtop wordt ons volgende doel?

Lieve paranimfen, Anouk en Michiel, wat fijn dat jullie me in deze laatste etappe willen bijstaan!

Michiel vanaf dag 1 van mijn onderzoek heb jij je over mij ontfermd. Zowel voor praktische als inhoudelijke dilemma's maakte je tijd voor me vrij in je overvolle agenda. Maar misschien belangrijker nog was er ook tijd voor onszelf als persoon: in het werk, in de muziek, op het hockeyveld of op het water, onze passie en ambities werden weer opgeladen.

Anouk, al was je wat verbaasd om als arts met weinig wetenschappelijke ambities als paranimf gevraagd te worden, ik denk toch dat je helemaal op je plaats bent. Onze jaren samen in Maastricht zijn gekenmerkt door een gezamelijk enthousiasme om voor studie en dispuut te gaan. Tegelijkertijd gaven we elkaar de vrijheid en ruimte om zaken te relativeren. Eén telefoontje met jou zet ogenschijnlijk belangrijke zaken weer heerlijk in perspectief!

Ik ben er trots op dat jullie op 9 september aan mijn zijde staan!

Lieve Guus en Nic, opgroeien met twee oudere broers is niet altijd even makkelijk, maar heeft toch ook zo zijn voordelen gebracht. Ik blijf dan wel voor altijd (het) 'kleintje', het heeft me ook gemotiveerd om af en toe boven jullie uit te stijgen. Veel dank dat dit altijd met veel liefde, humor en begrip gepaard is gegaan. Lieve Suus, wat heerlijk dat je voor Guus hebt gekozen en ons gezin extra warmte, gezelligheid, wat nuchterheid en natuurlijk Emilietje hebt gegeven.

Lieve mammie, al ben je er niet meer, ik weet dat je me ergens vanaf een wolkje of een bergtop in de gaten houdt en op het juiste moment je licht laat schijnen. Jouw onvoorwaardelijke liefde en geloof hebben me al zo ver gebracht, maar als jouw voorspelling klopt dan volgen er nog veel meer mooie dingen na het doctor-schap....

Lieve pappie, je bent de meest fantastische vader die een dochter zich zou kunnen wensen. Ik heb er inmens veel bewondering voor hoe jij je zonder mammie staande weet te houden. Ondanks het gemis van mammie heb je je vreugde in het leven behouden en heb je me in deze zware tocht volledig bijgestaan, zodat we straks kunnen genieten van het prachtige resultaat. Ik ben trots op je!

De bergen, winter of zomer, een omgeving waar wij ons naast de Friese meren helemaal thuis voelen. De plek waar wij letterlijk en figuurlijk de hoogtepunten uit ons leven beleven. Allerliefste Addy, je bent mijn steun en toeverlaat, de liefde van mijn leven, laten wij samen nog vele toppen bereiken….


Petit Combin (3672m)
– Grand Combin (4314m)

Curriculum vitae

CURRICULUM VITAE

Beatrijs Bloemers werd geboren op 23 mei 1980 te Utrecht en groeide op in Baarn met twee oudere broers. Na in 1998 haar eindexamen aan het Johan van Oldenbarnevelt Gymnasium te Amersfoort behaald te hebben, startte zij met de studie geneeskunde aan de Universiteit Maastricht. Tijdens haar studie was zij lid van de Maastrichtse Studentenvereniging Tragos en damesdispuut Topaas.

In 2002 deed Beatrijs haar wetenschappelijke stage bij de afdeling Kindercardiologie van het Wilhelmina Kinderziekenhuis onder leiding van Prof.dr. N. Sreeram, waarbij een deel werd doorgebracht bij de afdelingen kindercardiologie van de University of Washington School of Medicine, Seattle (VS) en de Oregon Health Sciences University, Portland (VS). Haar co-assistentschap Neurologie liep zij aan de Universiteit van Melbourne (Australië) en Kindergeneeskunde aan de Universiteit van Pretoria (Zuid-Afrika). Voor haar keuze co-assistentschap ging zij opnieuw naar de afdeling kindercardiologie van het Wilhelmina Kinderziekenhuis te Utrecht, waar zij ditmaal onder begeleiding van Dr. J. Strengers haar eerste ervaring als afdelingsassistent opdeed. Aansluitend aan het artsexamen was zij vanaf 1 februari 2005 als ANIOS werkzaam bij de kindergeneeskunde van het Wilhelmina Kinderziekenhuis (opleider Prof. Dr. J.L.L. Kimpen).

In het najaar van 2005 is Beatrijs naast het klinische werk gestart met een onderzoek naar RSV infecties bij kinderen met Down syndroom dat in 2006 resulteerde in een aanstelling als arts-onderzoeker bij de Divisie Kindergeneeskunde onder leiding van Dr. L.J. Bont en Prof. Dr. J.L.L. Kimpen. Haar promotieonderzoek resulterend in dit proefschrift werd verricht in samenwerking met het VU Medisch Centrum Amsterdam, onder begeleiding van Prof. Dr. A.M. van Furth.

Vanaf 1 augustus 2009 is Beatrijs werkzaam als ANIOS bij de afdeling Kindergeneeskunde van het Onze Lieve Vrouwe Gasthuis te Amsterdam (opleider Dr. A.A.M.W. van Kempen). Vanaf 1 september 2010 zal zij starten als ANIOS op Intensive Care voor kinderen van het Academisch Medisch Centrum te Amsterdam (opleider Prof. Dr. H.S.A. Heymans). Daar zal zij dankzij een subsidie vanuit het Dr. C.J. Vaillantfonds in samenwerking met Prof. Dr. A.P. Bos en Dr. J.B.M. van Woensel een vervolgonderzoek opstarten naar de effecten van beademing op luchtwegepitheel van kinderen met Down syndroom.

Beatrijs woont in Amsterdam, samen met Alexander Veldhuijzen, met wie zij in 2009 is getrouwd.

Setting new goals:
Cervin (4478m)

List of publications

LIST OF PUBLICATIONS

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