Adaptive immune system and vaccination responses in Down syndrome

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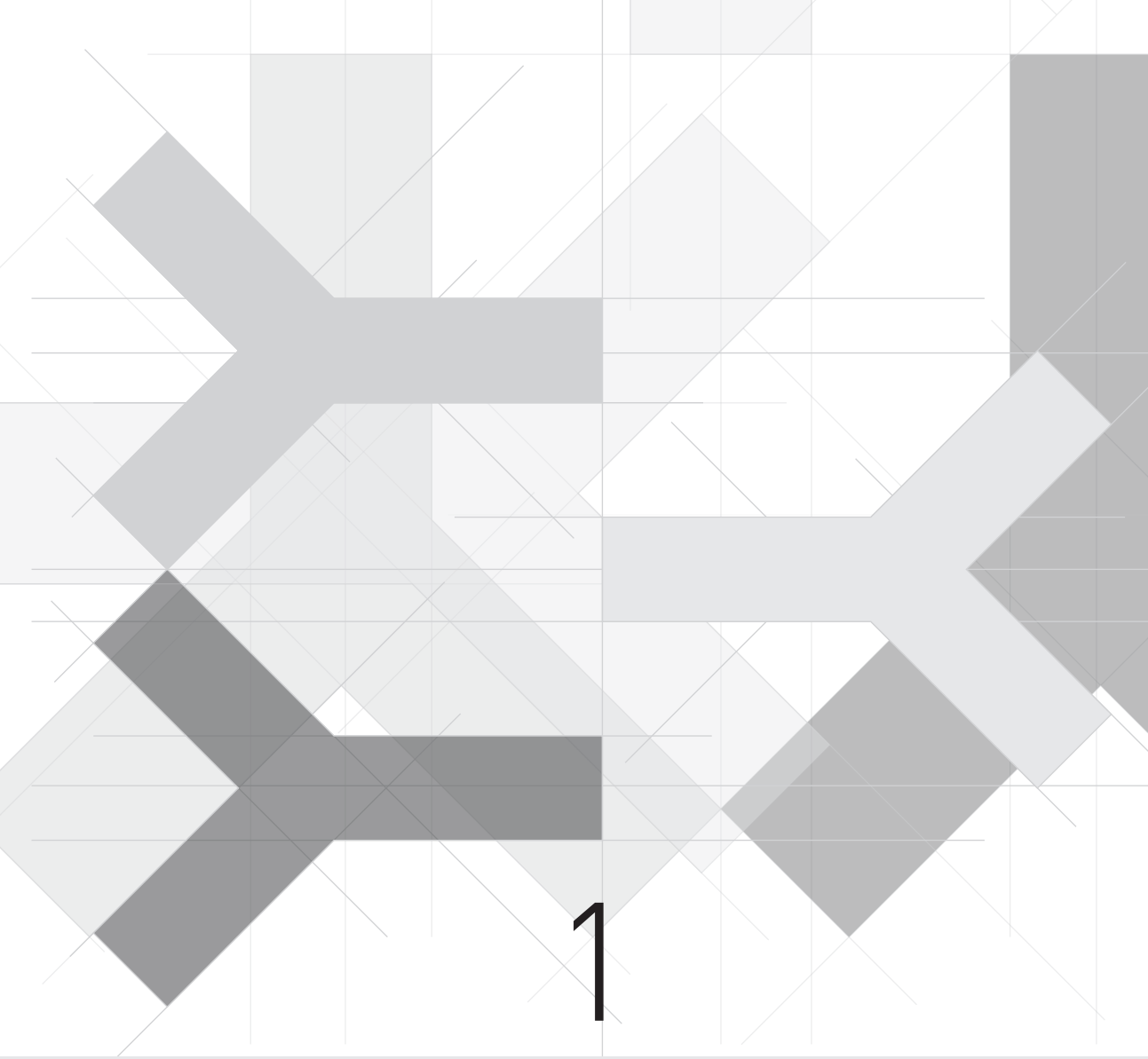
CONTENTS

Voor André

Sans toi, les émotions d'aujourd'hui ne seraient que la peau morte des émotions d'autrefois.

(Zonder jou zijn de gevoelens van vandaag slechts schilfers van die van vroeger)



1

GENERAL INTRODUCTION

Down syndrome (DS), named after John Langdon Down, is the most common chromosomal disorder in man. Down syndrome is caused by trisomy of (parts of) chromosome 21.[1] The incidence of Down syndrome in the Netherlands is around 14.6 per 10,000 live births, which results in around 245 live newborns with Down syndrome in the Netherlands each year.[2] Children with DS have distinctive phenotypic facial features such as oblique eye fissure, epicanthus, small ears, flat nasal bridge and a protruding tongue.

In addition to this, DS subjects show generalized hypotonia, short stature and mental retardation of variable severity. Around fifty percent has a congenital heart defect.[3] Children with DS have multiple ear-nose-throat abnormalities such as stenotic ear canals with smaller or abnormally inserted Eustachian tubes, and midfacial hypoplasia.[4] DS children also have hyperproduction of mucus and lower respiratory tract abnormalities such as laryngo- and tracheomalacia.

Clinical profile of infections, autoimmune disease and malignancies in DS

Nowadays, the majority of Dutch DS children live at home. They are exposed to the same viral and bacterial pathogens and environmental factors as their brothers and sisters. However, children with DS are more frequently ill.[5] They are more prone to respiratory tract infections. This is at least partly explained by a combination of their phenotypical facial features and their anatomical respiratory tract abnormalities together with generalized hypotonia. Complications of respiratory tract infections remain the most important cause of mortality in all age groups in DS.[6-10] DS children also show more haematological malignancies and autoimmune phenomena such as celiac disease, thyroid disease and diabetes mellitus.[11-15] Leukemia (both acute lymphoid and myeloid) and leukemoid reactions show an increased incidence in Down syndrome; estimates of the relative risk are 10-20x higher compared to the non-DS population.[11, 12, 16] Statistics on bacterial sepsis show higher morbidity and mortality figures in DS children.[6, 17] This combination of increased incidence of infections, autoimmune diseases and haematological malignancies has led to the hypothesis of an altered adaptive immune system in Down syndrome.

Several theories have been postulated. During corrective heart surgery it was noted that the DS thymus looked different compared to non-DS thymuses.[18-22] As a result, immunological research in DS focused on T-cell problems for decades. The clinical profile of recurrent infections, autoimmune diseases and malignancies in combination with a higher incidence of Alzheimer-like disease in relatively young DS adults led to the hypothesis of premature ageing or 'immunosenescence' of thymus and T-lymphocytes.[22-24] But the question remains: is that true? This thesis provides more in depth research - both quantitative and qualitative - on the alterations in the adaptive immune system in children with Down syndrome.

The adaptive immune system: basic background

The key players in the adaptive immune response are T- and B-lymphocytes. Both T- and B-lymphocyte precursors are generated from haematopoietic stem cells in the bone marrow. A unique B-cell antigen receptor is created and expressed on the membrane through gene rearrangements without previous antigen-exposure. While B-lymphocytes fully develop in the bone marrow, immature T-cell-precursors migrate to the thymus. Within the thymus, T-cell-precursors can only survive when their T-cell receptors can interact with self major histocompatibility complexes (MHC) expressed on cell membranes. Thymocytes binding to MHC-class I differentiate into cytotoxic-T-lymphocytes (Tc), thymocytes binding to MHC-class II differentiate into helper-T-lymphocytes (Th).

Naive Th-, Tc- and B-lymphocytes migrate to the secondary lymphoid organs (e.g. spleen, lymph nodes, gut- and mucosa-associated lymphoid tissue respectively GALT and MALT), and proliferate and differentiate into multiple different effector and memory subpopulations after antigen exposure.

B-lymphocytes react directly to antigen exposure by producing immunoglobulins (Igs). Extracellular pathogens such as bacteria are the main target of these Igs (humoral response).

Tc interact with antigen presented on MHC-class I molecules, which almost all human cells express, and can act directly as "killing machines". Tc are specifically suited for strong cellular immune responses against tumour cells and intracellular pathogens such as viruses.

Th can only interact with antigen-presenting cells (APCs) expressing MHC-class II molecules. Examples of APCs are B-lymphocytes, dendritic cells and phagocytes.

Th are responsible for coordination and communication with both innate and adaptive immune cells; in that sense they serve as immunoregulators. Th can help both humoral and cellular immune responses.

Lymphocyte distribution in the human body

Flow cytometric analysis using specific cell surface (cluster of differentiation, CD) markers can differentiate between the various naive and memory B- and T-lymphocyte subpopulations in peripheral blood. However, peripheral blood lymphocytes only represent around 2% of the total number of lymphocytes in the human body.[25] Lymphocytes continuously circulate through the body and migrate to their preferred sites situated in primary and secondary lymphoid organs. The majority of lymphocytes actually reside in the secondary lymphoid organs, especially in the lymph nodes (estimated 40% of total lymphocytes). So, by analysing peripheral blood, only a minor fraction of the lymphocyte population can be visualized (Figure 1). Therefore, conclusions based on peripheral blood analysis should be drawn with caution.

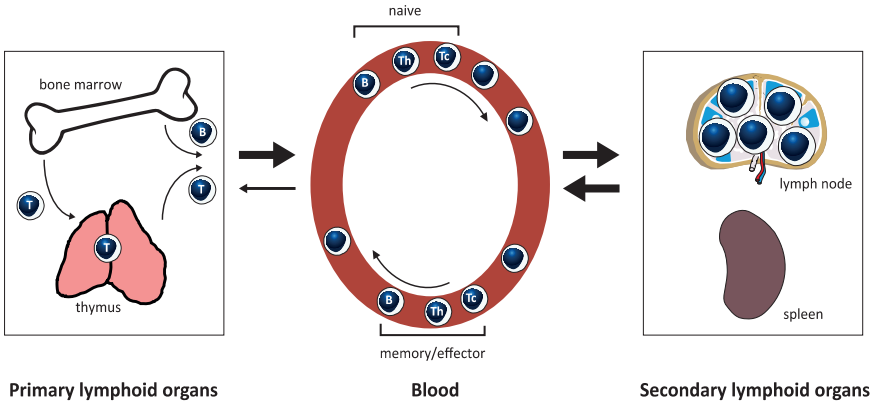


Figure 1 Analysing lymphocytes in peripheral blood represents around 2% of the total lymphocyte population, as the majority of lymphocytes reside in primary (bone marrow, thymus) and secondary lymphoid organs (such as lymph node and spleen).

Figure 1 is adapted from *Werkboek Immunologie* G.T. Rijkers et al.

Also, the distribution of lymphocytes can vary between individuals and groups.[26, 27] For instance in Down syndrome, an increased genetic expression of lymphocyte homing receptors and cell adhesion molecules is found in thymic epithelium and thymocytes, potentially leading to an inefficient release of T-lymphocytes into the peripheral blood and thereby alteration of the distribution of T-lymphocytes in the body. Moreover, the results of one specific blood analysis do not necessarily reflect a permanent difference, as many factors – e.g. recently encountered antigens – can strongly influence the amount of lymphocytes circulating in the blood.[27] Exposure to pathogens can also leave a personalised fingerprint, which shape is dependent on factors such as age at time of contact, season, environment, race, and sequence of encounters. Especially viruses trigger cytotoxic-T-lymphocytes to differentiate and proliferate to specific subpopulations. For example, after primary cytomegalovirus (CMV) contact, a persistent increase of CMV-specific terminally differentiated Tc-lymphocytes ($CD3^+CD8^+CD45RA^+CD27^-$) is seen; CMV leaves a typical fingerprint in the Tc population.[28] The individual set-point is defined by the degree of immunocompetence during the primary CMV-contact: immunocompromised people produce higher numbers of CMV-specific terminally differentiated Tc-lymphocytes in an attempt to keep CMV latent, but they are still more prone to CMV reactivation and severe infection-related morbidity.[29-31] The number of terminally differentiated Tc in combination with the clinical picture after primary CMV infection can therefore be

used as an indicator for an assessment of the immune status of the host during the encounter with CMV.

Vaccination types as models for T-cell (in)dependent antigen response

The adaptive immune system uses different pathways to respond to antigens: T-cell dependent (TD) and T-cell independent (TI), respectively. Different types of vaccination are used in this thesis as a model to study these different immune response pathways. All models have their limitations. A vaccine is not a surrogate for experiencing an infection for various reasons. The route is different: vaccinations are administrated intramuscularly in most cases, whereas pathogens invade across barriers (e.g. respiratory tract, skin) resulting in differences in antigen-presentation and immune response.

Also, the composition is different: vaccinations are made of attenuated live viruses, inactivated or killed organisms, inactivated toxins or segments of the pathogens. They can contain extra elements, such as a carrier protein (conjugate) or adjuvants to boost the immune response and push the immune response in a certain direction: e.g. a TI or TD antigen response. In reality, most pathogens share properties of TD and TI antigens.[32-34]

Finally, comparison is difficult: different methods and vaccination schemes co-exist side-by-side, but universal antibody cut-off levels are being used despite of the differences. The majority of the documented protective antibody cut-off levels are based on studies performed in healthy males (e.g. soldiers), but they are applied to children and elderly as well. Post-vaccination protection for an individual should be based not only on the quantitative antibody level, but also on the antibody quality measured by avidity and opsonisation capacity and most importantly the lack of disease occurrence. However, for most vaccines, combined quantitative and qualitative studies and reference values specifically for children are lacking.

T-cell dependent antigen response

The T-cell dependent antigen response is primarily determined by the combination of T-lymphocyte function, T-B interaction and B-lymphocyte function (Figure 2). An adequate T-cell dependent antigen response will ultimately lead to activated class-switched B-lymphocytes producing immunoglobulins with high affinity, and will also result in the formation of memory T- and B-lymphocytes.

Peptide antigen has to be processed and presented by an antigen-presenting cell (APC). Antigen-loaded APCs can then migrate to secondary lymphoid organs (e.g. lymph nodes, spleen) and present a specific antigen on their MHC-class II complex to the T-cell receptor (TCR) of helper-T lymphocytes (Th). If the MHC-class II-peptide complex is recognized by TCR of Th, an immunological synapse will be formed in the interface between APC and (B- or T-)lymphocyte. The formation consists of different

clusters of rings: in the centre, the B-cell or T-cell receptor which is bound to a MHC-antigen complex presented by APC, with surrounding clusters consisting of different adhesion molecules. For example in the peripheral supramolecular activation cluster (pSMAC), lymphocyte function-associated antigen 1 (LFA-1) on Th binds to intercellular adhesion molecule 1 (ICAM-1) on APC. The immunological synapse coordinates cell-signalling, activation and differentiation. The Th becomes activated and moves towards B-cell areas in secondary lymphoid organs.

B-lymphocyte activation is initiated following recognition of a specific antigen by the B-cell receptor (BCR). Activated B-lymphocytes can produce IgM instantly. The activated B-lymphocytes move towards the activated Th-lymphocytes in the secondary lymphoid organs and can present processed peptide-antigen on their MHC-II molecules to TCR of the abovementioned activated Th-lymphocytes.

With help from Th, B-lymphocytes can undergo class-switch recombination and somatic hypermutation. Apart from finding the right Th-B match, co-stimulatory molecules creating an immunological synapse between B-Th are necessary as well.

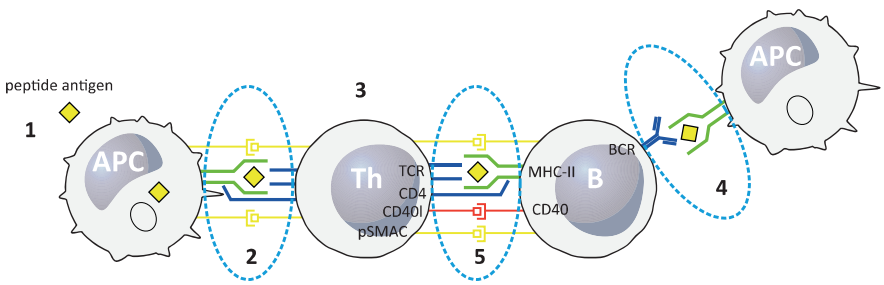


Figure 2 Stepwise approach of T-cell dependent antigen response.

1. Peptide antigen uptake and processing by antigen-presenting cell (APC).
2. Immunological synapse between APC-Th: antigen presentation on MHC-II by APC to TCR on helper-T-lymphocyte (Th). Antigen-recognition by TCR on Th. Cellular binding between Th and APC through costimulating peripheral supramolecular adhesion molecules (pSMAC) such as LFA-1 and ICAM-1, creating an immunological synapse.
3. Activation of Th and Th movement towards B-cell areas, generating cytokines activating both Th and APC.
4. Antigen-recognition of B-lymphocyte by BCR, antigen presentation by APC possible. Antigen-processing and presentation on MHC-II by B-cell. Movement of activated B-cell in lymph node towards Th.
5. Immunological synapse between B-Th: antigen-presentation on MHC-II of B-cell to TCR on Th. Antigen-recognition by TCR on Th. Cellular binding between Th and B-cell through costimulating peripheral supramolecular adhesion molecules (pSMAC) such as LFA-1 and ICAM-1, creating an immunological synapse. CD40-CD40L connection necessary for class switch recombination (CSR) of activated B-lymphocytes towards the production of IgG, IgA or IgE.

For instance, B-lymphocytes can only class-switch from the production of IgM to the production of IgG, IgA and IgE after interaction between CD40 molecules on B-lymphocytes with CD40L molecules on activated Th. Through this class-switch recombination (CSR) process, B-lymphocytes can adapt their Ig effector functions while maintaining antigen specificity.

Repeated exposure to T-lymphocyte dependent antigens activates selected clones of memory B-lymphocytes to undergo somatic hypermutation (SHM), leading to increased antigen specificity with higher affinity immunoglobulins. These processes also result in the production of memory B-lymphocytes inducing long lasting protection and faster immune responses when antigen is repeatedly encountered.[33, 34] Examples of TD antigens used in this thesis are tetanus toxoid and influenza A/H1N1 vaccinations.

T-cell independent antigen response

Bacteria can use camouflage techniques to prevent Th help, for instance by using a coat of polysaccharides. B-lymphocytes can mount an immune response against these polysaccharides without T-cell help, because these molecular structures are repetitive and therefore do not need peptides or antigen-presentation on MHC to activate the B-lymphocyte. This is called a T-cell independent (TI) type 2 immune response (Figure 3a). Polysaccharides can extensively crosslink B-cell antigen receptors and deliver a prolonged and persistent signal to the B-lymphocyte.[32] TI immune response is essential for rapid antibody production and early protection especially to blood-borne pathogens. However, without Th help, no B-lymphocyte class switch recombination or somatic hypermutation will take place and no memory B-lymphocytes will be produced. The TI immune response can be used to investigate the maturation and quality of B-lymphocytes. An example of a specific TI type 2 antigen used in this thesis is 23-valent pneumococcal polysaccharide vaccine (PPV23).

The immature adaptive immune system in infants under 2 years of age is unable to induce an adequate TI response in response to polysaccharides. To overcome this problem, new types of vaccines have been developed. By conjugating a peptide to the polysaccharide antigen, an adequate TD immune response can be induced, even in young children (Figure 3b). Examples of this type of immune response used in this thesis are the heptavalent pneumococcal conjugate vaccine (PCV7) and meningococcal serotype C (MenC) vaccine.

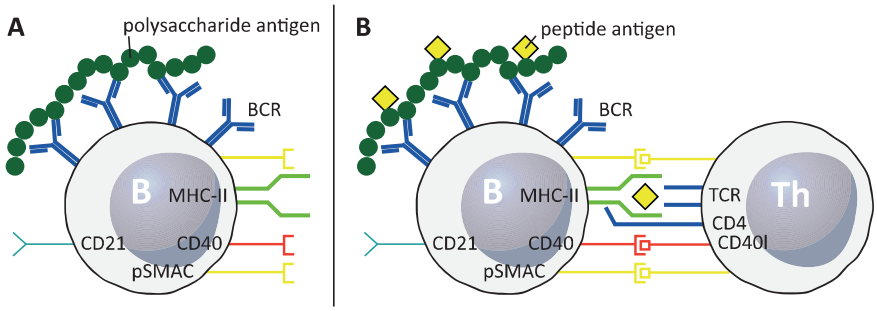


Figure 3 Stepwise approach of T-cell independent antigen response (polysaccharide PS) and PS+conjugate.

A. PS: Extensive polysaccharide crosslinking on BCRs: B-cell activation signal 1. Extra activation through C3d recognition by CD21 on B-cell. Extra stimulation through cytokines produced by macrophages/T-lymphocytes possible. TI antigen response: primarily IgM.
B. PS with conjugate: Th help possible through conjugate (peptide) uptake and processing by B-lymphocyte. Peptide antigen-presentation on MHC-II of B-cell to TCR on Th. Costimulating peripheral supramolecular adhesion molecules (pSMAC) creating immunological synapse, connection CD40-CD40L necessary for class switch recombination (CSR) of B-lymphocytes. Th activation: effector and memory cells, cytokine production. TD antigen response: primarily IgG.

THESIS OUTLINE

This thesis gives more insight in the adaptive immune system of children with Down syndrome. In **PART 1** both T- and B-lymphocytes subpopulations and immunoglobulin (IgG, IgA, IgM) levels in different age groups in DS children are analyzed. In **Chapter 2,** an overview of past literature on the DS adaptive immune system is given. **Chapter 3** investigates the current hypothesis of immunosenescence by comparing the literature on immunological alterations and clinical profiles in normal ageing, progeria syndromes and DS. In **Chapters 4 and 5** both T- and B-lymphocyte subpopulations and immunoglobulin (IgG, IgA, IgM) levels in different age groups in DS children are analyzed and compared with healthy age-matched control children. Terminally differentiated cytotoxic-T-lymphocyte counts in relation to cytomegalovirus are compared with non-DS subjects with different immunocompetence status. The clinical picture of infections, autoimmune disease, allergy and malignancies is correlated with these quantitative results. In **PART 2** the quality of B- and T-lymphocyte responses to different types of antigen is investigated through vaccination studies as a model for TD and TI immune responses. In **Chapters 6-9** five different vaccinations

are used. Tetanus toxoid (chapter 6), influenza A/H1N1 (chapter 7) are examples of protein antigens eliciting TD responses. Meningococcal C (MenC; chapter 8) and heptavalent pneumococcal conjugate (PCV7; chapter 9) vaccinations are examples of protein-conjugated polysaccharide antigens eliciting TD responses. 23-Valent pneumococcal polysaccharide vaccine (PPV23; chapter 9) is an example of a polysaccharide antigen eliciting a TI response. A comparison is made with data from the literature on ageing, on DiGeorge syndrome (DGS), thymectomy and HIV (all mainly T-lymphocyte deficiencies), and on common variable immunodeficiency disorders (CVID) and specific polysaccharide antibody deficiency (SPAD) (mainly B-lymphocyte immunodeficiencies). **PART 3** contains the summary and discussion.

AIM OF THE THESIS

In this thesis the following questions are addressed:

1. What alterations - both qualitative and quantitative - can be found in T- and B-lymphocytes in DS?
2. Do these alterations fit the current hypothesis of early immunosenescence?
3. Do children with DS have an intrinsic primary immunodeficiency (PID)?
4. Do the immunological alterations in combination with the clinical profile fit a specific known PID pattern or do children with Down syndrome have their own profile?

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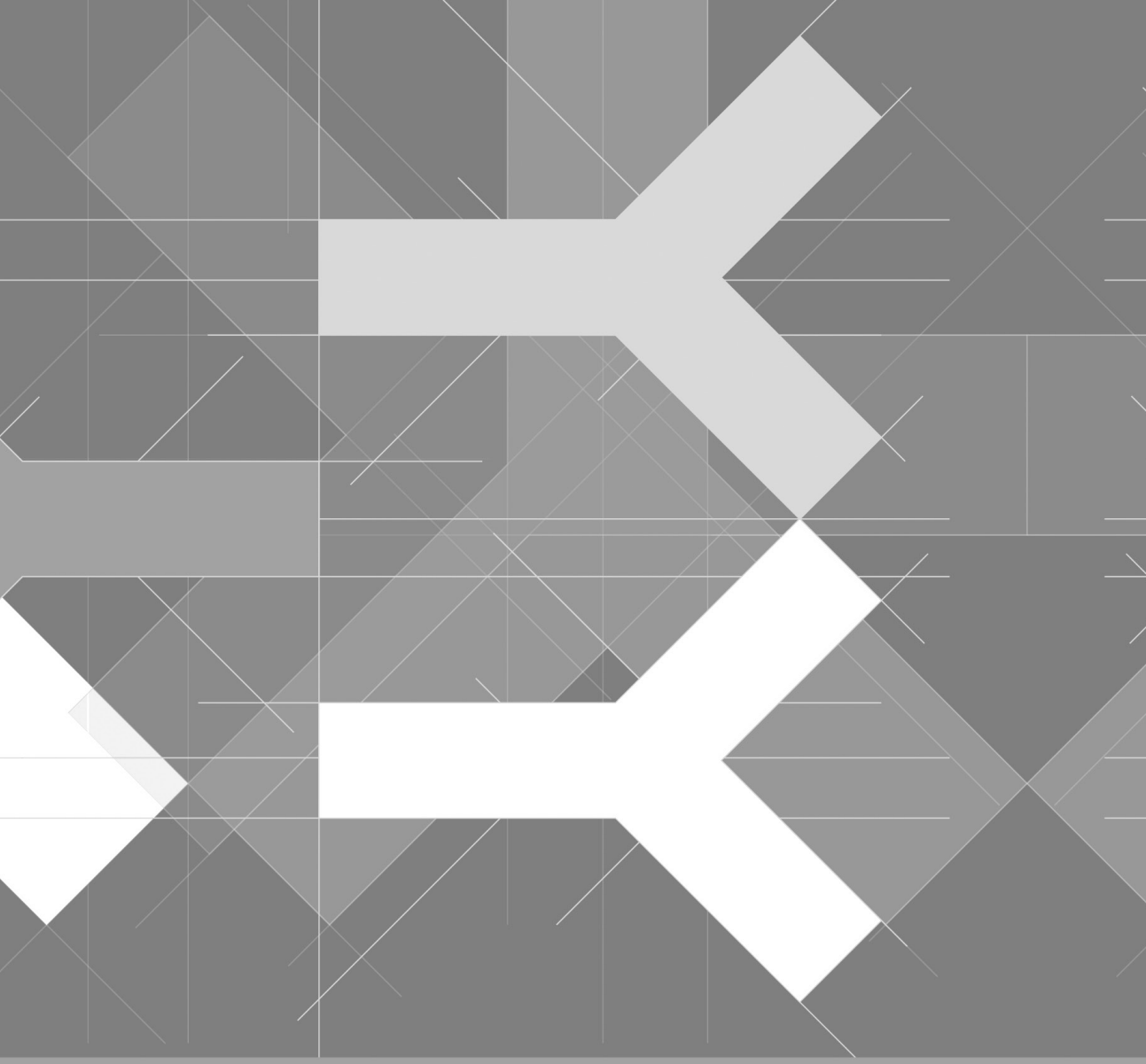

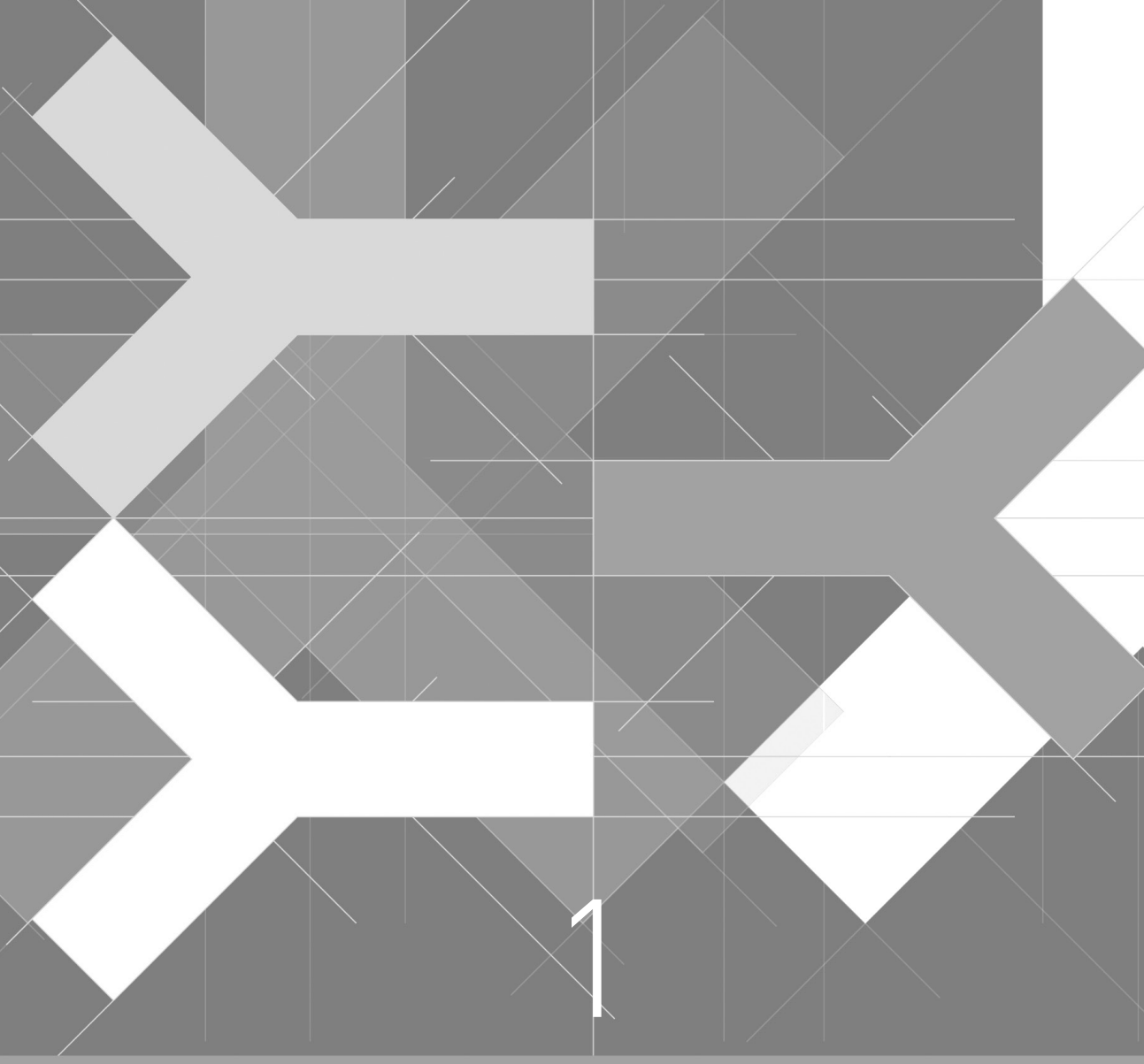

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LYMPHOCYTE SUBPOPULATIONS AND IMMUNOGLOBULIN PROFILES

2

INTRINSIC DEFECT OF THE IMMUNE SYSTEM IN CHILDREN WITH DOWN SYNDROME; A REVIEW

MAA Kusters, RHJ Verstegen, EFA Gemen, E de Vries

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ABSTRACT

Down syndrome (DS) is the most frequent cause of mental retardation in man. Immunological changes in DS have already been observed since the 1970s. The neurological system appears to be ageing precociously, with early occurrence of Alzheimer disease; until now, the observed immunological differences have been interpreted in the same context. Reviewing past and present results of immunological studies in DS children in relation to the clinical consequences they suffer, we conclude that it is more likely that the DS immune system is intrinsically deficient from the very beginning.

INTRODUCTION

Down syndrome (DS), or trisomy 21, is the most frequent genetic cause of mental retardation in man; the incidence is approximately 1 in 750 live births [1]. Consequently, doctors frequently see patients with DS and encounter their complex medical problems. Individuals with DS are invariably cognitively impaired, although the severity is highly variable. Characteristic facial features and hypotonia are present in almost all patients; around 50% suffer from congenital cardiac anomalies. Congenital cataract, abnormalities of the gastro-intestinal tract and orthopaedic, eye and ear problems occur with increased frequency compared with non-DS individuals. Histopathologic studies show a small and hypocellular brain and, by the fourth decade, characteristic features of Alzheimer disease [2, 3]. Autoimmune phenomena such as acquired hypothyroidism, celiac disease and diabetes mellitus occur in higher frequency compared with non-DS subjects. Leukaemia is estimated to be 15-20 times more frequent in DS [4-7]. Despite advances in treatment, infections - especially pneumonia - and leukaemia are still major causes of morbidity and mortality in DS [7-12]. The increased frequency of haematological malignancies, autoimmune diseases and infections in DS, and the observed high frequency of hepatitis B surface antigen (HBsAg) carriers, had already led in the 1907s to the hypothesis that DS is associated with abnormalities of the immune system [4, 13-16]. Indeed, many differences between the immune system of DS and non-DS individuals have been found throughout the years, and several hypotheses have been formulated which, if true, could have consequences for everyday clinical care in DS (findings relevant for everyday clinical care are summarized in Table 1).

Higher rates of infections, malignancies, and autoimmune phenomena are seen normally in elderly individuals [17-20], and DS was therefore hypothesized to be a form of abnormal precocious aging in various papers published in the late 1980s and early 1990s (e.g. [21-22]), which are still being cited (e.g. [23]).

Natural killer cells and innate immunity

The supposedly higher percentages of natural killer (NK) cells found in DS seems to support this theory of precocious aging [21, 24], as high percentages of NK cells are seen normally with ageing. However, these studies were performed in small groups of DS individuals with single- and double-colour flowcytometric staining techniques that could not differentiate between NK cells ($CD3^-$) and NK marker-bearing T-lymphocytes ($CD3^+$). Our recent study on lymphocyte subpopulations in DS shows lower absolute numbers of $CD3^-$CD16 and/or 56^+ NK cells in all age groups [25]. Populations with different NK-activity, capable of low, intermediate and high cytotoxicity against the NK-sensitive tumour cell line K562, respectively, were described

Table 1 Overview of differences relevant to everyday clinical care found between the immune systems of DS and non-DS individuals since the 1970s.

Lymphocyte subpopulations		Reference
$CD3^-CD16and/or56^+$ NK-cells	Decreased (abs)	[25]
$CD19^+$ B-lymphocytes	Decreased (abs; %)	[24, 25]
$CD3^+$ T-lymphocytes	Decreased/normal (abs)	[25]
$CD3^+CD4^+$ helper-T-lymphocytes	Decreased (abs; %)	[25]
$CD3^+CD8^+$ cytotoxic T-lymphocytes	Decreased/normal (abs)	[25]
$CD4^+CD45RA^+$ cells	Decreased (%)	[37, 38]
Th1/Th2 ratio	Increased	[69]
CD4/CD8 ratio	Inverted ratio	[22]
TCR-$\alpha\beta^+$ T-lymphocytes	Decreased (%)	[37]
$CD8^+CD57^+$ cells	Increased (%)	[21]
Immunoglobulins		
IgG	Increased >6yr	[15, 28, 30]
IgM	Decreased >6yr	[15, 28]
IgA	Increased >6yr/normal	[15, 30]
IgG_1	Increased/normal	[30, 70]
IgG_2	Decreased/normal	[30, 70]
IgG_3	Increased/normal	[30, 70]
IgG_4	Decreased/normal	[30, 70]
Response to vaccination		
Pneumococcal polysaccharide vaccine	Decreased/normal	[28, 54]
Tetanus vaccine	Decreased	[74]
Pertussis vaccine (acellular)	Decreased	[58]
Hepatitis B vaccine	Decreased/normal	[52, 57, 60]
Hepatitis A vaccine	Normal	[56]
Influenza vaccine	Decreased	[74]
Polio vaccine (oral)	Decreased	[59]

Abs, absolute counts; CD, cluster of differentiation; Ig, immunoglobulin; NK, natural killer; TCR, T cell receptor; Th, helper-T-lymphocyte; %, relative counts.

in the 1980s [26, 27]. Several authors describe a significant increase of cells possessing the low NK-activity phenotype in DS, associated with a significant decrease of cells with the intermediate and high NK-activity phenotype [21, 28]. With longevity, however, NK cells with well-preserved cytotoxic function increase [29].

Thymus and T-lymphocytes

The thymus is smaller in DS subjects, even in newborns, and has an abnormal structure [16, 26, 28, 30-32]. This suggests that T-lymphocytes are the core of the problem in DS; however, children with congenital heart disease who require cardiac surgery with (partial) thymectomy show rapid and permanent changes in T-lymphocyte numbers [33, 34] but, unlike in DS, their frequency of infections and autoimmune diseases is not increased [35]. The DS thymus shows a decreased proportion of phenotypically mature thymocytes expressing high levels of the αβ-form of the T-cell-receptor (TCR-αβ) and associated CD3-molecule [36], and overexpression of tumour necrosis factor (TNF)-α and interferon (IFN)-γ cytokines [27]. Overexpression of these cytokines suggests a dysregulation in cytokine production in DS and may provide an explanation for the abnormal thymic anatomy and thymocyte maturation [27]. An increased percentage of peripheral T-lymphocytes expressing the alternative γδ-form of the T-cell receptor (TCR-γδ) has been reported [26, 37], as well as a lower percentage of $CD4^+CD45RA^+$ naive cells – then considered to represent cells that have recently emigrated from the thymus - and a higher percentage of $CD29^+$ memory cells [26, 38]. TCR excision circle (TREC) counts are used to estimate recent thymic emigrants (VDJ recombination events excise intervening stretches of DNA) [39]. A significantly lower number of $TREC^+$ peripheral blood cells is found in DS children in comparison with healthy control children [23, 40]. These findings could be interpreted as early senescence of the immune system [26, 38], because naive helper- and cytotoxic T-lymphocytes [29, 41] as well as $TREC^+$ peripheral blood cells [42] decrease with ageing, while central and effector memory helper-T-lymphocytes and effector memory and terminally differentiated cytotoxic T-lymphocytes increase [43]. We have recently demonstrated a T-lymphocytopenia in all age groups, however, not just in older DS children, that concerns $CD4^+$ helper- as well as $CD8^+$ cytotoxic T-lymphocytes with absence of the tremendous expansion that is seen normally in the first year of life, suggesting a deficient reaction to antigenic stimulation [25, 41, 44]. Absolute numbers of T-lymphocyte populations gradually approach those of normal children over time [25], but it is doubtful whether these cells have normal phenotype and function, having shown a lack of the antigen-driven expansion in earlier years. Functional abnormalities of T-lymphocytes that have been described support this: the *in vitro* proliferative response to phytohemagglutinin (PHA), is markedly below normal in DS infants and adults [15, 16, 45-47]. In addition, bacterial and viral antigen-induced *in vitro* interleukin-2 (IL-2) production is reduced markedly, although PHA-stimulated IL-2 production is not impaired [13, 42, 43]. An interesting hypothesis is that overexpression of the cell adhesion molecules lymphocyte function-associated antigen-1 and DS cell adhesion molecule - located on chromosome 21 - causes higher affinity between cells leading to abnormal maturation and function [48, 49], but in most genetic studies in trisomy 21 an overall 150% increase of gene expression is not seen; the genetic overexpression is

often specific for a particular organ [50]. Enhanced cell death by apoptosis could also play a role, as transgenic copper-zinc superoxide dismutase mice (in humans located on chromosome 21) show enhanced apoptosis [51].

B-lymphocytes and antibody production

A considerable hypergammaglobulinemia of immunoglobulin (Ig)G and IgA after the age of 5 years, with high levels of IgG_1 and IgG_3 and low levels of IgG_2 and IgG_4, is described in DS [15, 30, 52], with IgM levels decreasing in adolescence. IgD levels are high [53]. Antibody responses to rabbit erythrocytes and Escherichia coli antigens are low [28], as are the responses to vaccine antigens such as influenza A, oral polio, acellular pertussis, tetanus and polysaccharide pneumococcal vaccine [54-59]. The frequency of hepatitis B virus carriers is much higher among DS children compared with age-matched controls; however, normal responses to hepatitis A and B vaccinations are seen, although specific IgG-subclasses can vary [56, 60]. Autoantibodies against human thyroglobulin and gliadin are observed more often in DS children [15, 30, 61], as are high titres against casein and beta-lactoglobulin [15, 61].

Somewhat paradoxically, we have recently found a profound B-lymphocytopenia in DS, with absence of the normal enormous expansion in the first year of life [25]. This has been described before [24, 28, 62, 63], but so far has attracted little attention. Recent observations even show a significant decrease of B lymphocytes ($CD19^+$) in fetuses with DS [64]. These abnormalities can be either due to an intrinsic B-lymphocyte defect or to the consequence of deficient helper-T-lymphocyte function causing inadequate control of B-lymphocyte activation and proliferation. The combination of profound B-lymhocyto*penia* and *hyper*gammaglobulinemia suggests the latter, with the possibility that antibody responses may be oligoclonal and/or inadequate in DS. However, we have found no mono- or oligoclonal M-proteins in 88 DS children (unpublished data). Also, in comparison, patients with DiGeorge syndrome (DGS; 22q11-deletion) show a congenital thymic hypoplasia with a variable degree of T-lymphocyte deficiency in 80% of cases [65,66]. As in DS, $TREC^+$ cell counts are decreased in the periphery, and T-lymphocytes gradually approach normal numbers over time [39] but – unlike in DS – B-lymphocytopenia is not seen in DGS [67, 68].

Helper-T-lymphocyte type 1 cells (Th1) produce cytokines such as IFN-γ, IL-2, and TNF-α which stimulate cytotoxic T-lymphocyte responses and IgG_1 and IgG_3 production, whereas helper-T-lymphocyte type 2 cells (Th2) produce cytokines such as IL-4, IL-5, IL-6, and IL-10 which stimulate antibody responses by B-lymphocytes and the formation of IgG_2 and IgG_4. In comparison to individuals with mental retardation (no DS) and healthy controls, DS adults have significantly higher percentages of IFN-γ-producing $CD4^+$ and $CD8^+$ cells and a higher Th1/Th2 ratio [69]. This fits the increased levels of IgG_1 and IgG_3 and decreased levels of IgG_2 and IgG_4 in DS, and supports disturbed helper-T-lymphocyte function [30, 70].

Clinical presentation in relation to immunodeficiency

The clinical presentation of DS children, seen in relation to possible immunodeficiency [71], is dominated by recurrent ear-nose-throat (ENT) and airway infections in their early years, followed by an increasing frequency of autoimmune diseases and lymphoproliferation thereafter. The recurrent ENT and airway infections could fit antibody deficiency, although the macroglossia, hypotonia, and altered anatomy of the upper airways will also play an important role in these infants. The tendency towards autoimmune diseases and lymphoproliferation, on the other hand, points primarily to immunodysregulation. Partial reduction in the number and function of T-lymphocytes can disturb the tolerogenic balance, generating a combination of immunodeficiency and immune dysregulation [72, 73]. DS children as a group could fit the picture of primary immunodeficiency, but with apparent individual differences. The relation between the abnormality of immunological values in individual DS children and the clinical complications has, so far, unfortunately not been studied extensively.

CONCLUSION

In summary, it is much more likely that the immune system in DS is intrinsically deficient from the very beginning, and not simply another victim of a generalized process of precocious ageing. It is not yet clear but at least possible that, next to the apparent thymus and T-lymphocyte abnormalities in DS, B-lymphocytes are also intrinsically different.

Further studies are needed to resolve the underlying mechanisms of this immunodeficiency, and to assess the implications thereof for everyday clinical care.

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3

DOWN SYNDROME: IS IT REALLY CHARACTERIZED BY PRECOCIOUS IMMUNOSENESCENCE?

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ABSTRACT

The immune system declines with ageing, leading to an increased susceptibility to infections and higher incidence and progression of autoimmune phenomena and neoplasia. Down syndrome prematurely shows clinical manifestations that are normally seen with ageing. This review provides a concise overview of abnormalities in the adaptive immune system of Down syndrome in comparison to normal and precocious (Progeria syndromes) ageing. Clinical signs, immunological changes and genetics are reviewed. We challenge the hypothesis that the immunological abnormalities in Down syndrome should be interpreted as precocious immunosenesence.

INTRODUCTION

In humans, as in all species, a progressive functional decline occurs with ageing in all organ systems due to loss and instability of genetic material. To protect the genome, chromosomes are capped by so-called telomeres which prevent degradation of genes near the ends of the chromosome. But with each cell division a small part of the telomere is lost. This proces is limited, but not prevented, by the enzyme telomerase which increases the length of the telomeres. Therefore, cumulative cell divisions ultimately result in genome instability and apoptosis of cells [1]. The immune system is essential for detection and elimination of pathogens and thereby for prevention of damage and degeneration of the organism. Lymphocytes continuously undergo proliferation [2]. So, especially lymphocytes are highly susceptible to telomeric shortening, leading to ageing of the adaptive immune system, which is often referred to as 'immunosenescence'. As a result, the elderly are more susceptible to infections. Also, malignancies and autoimmune phenomena are more common with ageing due to failing immune surveillance secondary to immunosenescence [3, 4].

Down syndrome is the most frequent genetic cause of mental retardation in man; it is caused by an extra chromosome 21. People with Down syndrome (DS) prematurely show signs that are normally seen with ageing; for example, adults with DS show early signs of Alzheimer's disease [5]. Also, clinical features reminiscent of immunosenescence are seen, with higher rates of infections, malignancies, and autoimmune phenomena. It is therefore not illogical to hypothesize that the immune system in DS shows accelerated ageing as well [6].

This review provides a concise overview of the abnormalities found in the DS immune system in comparison to normal immunosenescence and the immunological findings in the precocious aging or Progeria syndromes (PS) [7]. The scope of this article is not exhaustive with respect to this complex topic, but attempts to show that the immune alterations in DS should – in spite of previous publications on the subject – *not* be interpreted as precocious immunosenescence [8].

The adaptive immune system

The immune system defends the human body against invading micro-organisms. It consists of two parts: the innate and the adaptive immune system. The innate immune system provides a primary immune response. Although a fast reaction takes place within minutes to hours, no memory is generated. The innate immune system therefore provides only short-term solutions. The adaptive immune system plays a pivotal role for long-term survival.[9] An essential difference between innate and adaptive immune cells is that the latter react specifically to a myriad of antigens while

providing long-term memory as well. Since immunoscenesence primarily affects the adaptive immune system, this system will be the focus of this review.

Key players in the adaptive immune response are B- and T-lymphocytes. B-lymphocytes are responsible for humoral immunity by producing specific antibodies. T-lymphocytes are accountable for cellular immune responses by helping other immunological cells through cytokine production and stimulation, and by direct cytotoxicity. Both T- and B-lymphocyte precursors are generated from hematopoietic stem cells in the bone marrow. While B-lymphocytes fully develop in the bone marrow, T-cell-precursors migrate to the thymus for further proliferation and development. In the secondary lymphoid organs (spleen, tonsils, lymph nodes) antigens are collected and presented. Also, T- and B-lymphocytes migrate there, and proliferate and differentiate into different effector and memory subsets after stimulation.

Within the thymus, T-cell-precursors can only survive if their T-cell receptors can interact with self major histocompatibility complexes (MHC) expressed on cell membranes, so-called positive selection. Too strong binding to self-antigens leads to cell death by negative selection, no binding at all results in cell death by neglect. Thymocytes binding to MHC-type II differentiate into helper-T-lymphocytes (Th), thymocytes binding to MHC-type I differentiate into cytotoxic-T-lymphocytes (Tc). As only antigen-presenting cells such as B-lymphocytes, dendritic cells and phagocytes express MHC-type II molecules, Th can only interact with these types of cells. Th are responsible for coordination and communication with both innate and adaptive immune cells; they serve as immunoregulators. Tc interact with MHC-type I expressing cells, which almost all human cells are, and can act directly as “killing machines” after activation and proliferation. Tc are especially suitable for strong cellular immune responses against tumour cells and intracellular pathogens such as viruses, whereas Th can help both humoral and cellular immune responses. The continuous generation of new Th and Tc from the thymus is crucial to maintain a functional immune system.

Primary B-cell development takes place in the bone marrow. A unique B-cell antigen receptor is created on each B-lymphocyte membrane through gene rearrangements without previous antigen-exposure. B-lymphocytes do not need MHC for antigen recognition and can respond not only to peptides, but also to polysaccharides. Naive B-lymphocytes react to antigen exposure by producing immunoglobulins (Igs), primarily IgM. Extracellular pathogens such as bacteria are the main focus for these Igs. T-lymphocytes and T-lymphocyte-derived factors are necessary for further B-lymphocyte development. With the help of Th, B-lymphocytes can class-switch to the production of IgG, IgA and IgE, with altered effector function while maintaining antigen specificity. Repeated exposure to T-lymphocyte dependent antigens activates

selected clones of memory B-lymphocytes to undergo somatic hypermutation (SHM) leading to higher affinity Igs.

The net result of all these processes is a broad diversity of B- and T-lymphocytes, which can survive for many years and provide resistance against the pathogens attacking the human body.

Down syndrome compared to normal ageing

A comparison between the adaptive immune systems of Down syndrome, normal ageing and PS is summarized in Table 1.

T-lymphocytes

The continuous release of new Th and Tc from the thymus is crucial for maintaining a functional immune system. Recent thymic emigrants all carry T-cell receptor rearrangement excision circles (TREC) as a by-product of DNA recombination processes. TRECs are not replicated and therefore diluted in the progeny that is formed after cell division. The TREC content can therefore be used to estimate the thymic output and also – indirectly – to estimate the thymic involution with ageing.

With ageing the renewal capacity of stem cells declines, the hematopoietic tissue in the bone marrow decreases, and thymic involution with low peripheral blood TREC counts ensues [1]. T-lymphocytes can influence their own differentiation and proliferation process in the thymus and periphery by cross-talk and feedback-mechanisms. Decreased output of thymic emigrants can therefore normally be compensated in ageing individuals by an increase in effector and memory Th and Tc numbers. In this way, total T-lymphocyte counts remain relatively stable in ageing adults despite decreasing naive counts, as effector and memory subsets fill up the T-lymphocyte pool [3, 4]. However, these T-lymphocytes are continuously antigen-driven and eventually accumulate in "dead end" T-lymphocyte subsets such as terminally differentiated (TD) Tc. In other words, the T-lymphocyte pool becomes more experienced but less flexible with ageing, and the cells show a restricted repertoire and reduced proliferative response [3, 4].

People with DS show T-lymphocyte abnormalities from an early age onwards. Newborns and fetuses with DS already show an altered thymic anatomy with impaired thymic output and lower TREC counts [10]. Some interesting candidate genes influencing thymocyte production by altered cross-talk and feedback-mechanisms can be found on chromosome 21. For example, increased expression of DS-cell-adhesion molecules on thymic epithelia and thymocytes can cause abnormal T-lymphocyte maturation and inefficient T-lymphocyte release to the peripheral blood [11]. Naive Tc and Th are decreased from birth [18]. Apart from decreased production, proliferation is impaired in DS as well. Tc and Th lack the normal antigen-driven expansion in the

Table 1 The adaptive immune system in normal ageing, Progeria syndromes and Down syndrome.

	Normal ageing	Progerias	Down syndrome
References	**[1,3,4,22-24,29]**	**[7,37-43]**	**[5,6,8,10-13,19-21,25-28,30-36]**
T-lymphocytes			
$CD3^+$ T-lymphocytes	Decreased (abs)	HGPS: decr/normal (abs) CS, Werner, XP: normal (abs/rel)	Decr/normal (abs)
$CD3^+CD4^+$ Th	Decreased (abs)	HGPS: decr/normal (abs) CS, XP: normal (abs)	Decreased (abs)
$CD3^+CD8^+$ Tc	Decreased (abs)	Normal (abs)	Decr/normal (abs)
$CD4^+CD45RA^+$ (Naive Th)	Decreased (abs)		Decreased (abs)
$CD4^+CD45RO^+$ (Memory Th)	Increased (abs)		Normal (abs)
$CD8^+CD45RA^+CD27^+$ (Naive Tc)	Decreased (abs)		Incr/normal (abs)
$CD8^+CD45RA^+CD27^-$ (Term Diff Tc)	Increased (abs)		Normal (abs)
T-lymphocyte proliferation	Decreased	CS:normal HGPS:decr/normal Werner:decr/normal XP:decr/normal	Decr/normal
Thymus	Abnormal structure		Smaller, abnormal structure
TREC count	Decreased		Decreased
Th1/Th2 ratio	Normal		Increased
CD4/CD8 ratio	Normal	Decreased	Decreased
B-lymphocytes			
$CD19^+$ B-lymphocytes	Normal (abs)	HGPS: incr/normal (abs) Werner: normal (rel)	Decreased (abs)
$CD19^+CD27^-$ (Naive B)	Decreased (abs)		Decreased (abs)
$CD19^+CD27^+$ (Memory B)	Increased (abs)		Normal (abs)
B-lymphocyte proliferation (PWM)	Decreased	HGPS: incr/normal Werner: decr/normal	Decreased

Immunoglobulins			
IgG	Decr/normal	HGPS: decr/normal Werner: normal	Increased >2yr
IgM	Normal	Normal	Decreased
IgA	Decr/normal	HGPS: decr/normal Werner: normal	Normal
IgG_1	Normal		Increased > 3yr
IgG_2	Normal		Decreased
IgG_3	Normal		Incr/normal
IgG_4	Normal		Decreased
Auto-antibodies	Increased	HGPS: no increase	Increased
Oligo/monoclonal antibodies	Increased		No increase
Escherichia coli antigen	Decreased response		Decreased response
Response to vaccines			
Tetanus	Decreased		Decreased avidity
Pneumococcal polysaccharide vaccine	Decreased		Decr/normal
Meningococcal conjugate vaccine	Decreased		Decreased
Pertussis vaccine (acellular)	Decreased		Decreased
Hepatitis B vaccine	Decreased		Decr/normal
Hepatitis A vaccine	Decreased		Normal
Influenza vaccine	Decr/normal		Decr/normal
Polio vaccine (oral)	Normal		Decreased
Response to viruses			
CMV	Increased specific T-subsets, increased reactivation		Normal
RSV	Increased risk		Increased risk and severity

Table 1 Continued.

	Normal ageing	Progerias	Down syndrome
References	[1,3,4,22-24,29]	[7,37-43]	[5,6,8,10-13,19-21,25-28,30-36]
Response to bacteria			
Bacterial sepsis	Increased rate	HGPS, Werner: increased rate	Increased rate
Mortality/morbidity	Increased rate	Increased rate	Increased rate
Auto-immune diseases			
Thyroid	No increase	No increase	Increased
Diabetes Mellitus	Increased type 2	Incr/normal type 2	Increased type 1
Celiac disease	No increase		Increased
Malignancies			
Hematological	Increased	XP: possible increase	Increased
Non-hematological	Increased	Werner: increased XP: increased skin cancer	No increase
Age-related diseases			
Osteoporosis	Increased	Increased	No increase
Atherosclerosis	Increased	Increased	No increase
Alzheimer disease	Increased	Increased	Increased
Periodontal disease	Increased		Increased

Abs = absolute counts; CD = cluster of differentiation; CMV= cytomegalovirus; CS= Cockayne syndrome; decr= decreased; HGPS = Hutchinson-Gilford progeria syndrome; Ig = immunoglobulin; Incr=increased; PWM = pokeweed mitogen; rel= relative counts; RSV = respiratory syncytial virus; TCR = T-cell-receptor; Tc= cytotoxic-T-lymphocyte; Term Diff = terminally differentiated; Th = helper-T-lymphocyte; TREC = T-cell receptor rearrangement excision circles; XP= xeroderma pigmentosum; yr = years. Empty column = not described in current literature.

first years of life. Memory Tc show a gradual increase over time, but in contrast to normal ageing memory Th do not. The expansion of TD Tc with normal ageing is not seen in DS either [12].

The continuous DNA rearrangement processes make the lymphocyte pool extremely vulnerable to DNA errors. Lymphocytes are capable of upregulating telomerase and can thereby prolong their lifespan [1, 2]. The constant microbial pressure throughout life leads to an ongoing proliferative demand, which ultimately results in genome instability, senescence and apoptosis of cells. Enhanced cell death by apoptosis could play an extra role in DS besides decreased T-lymphocyte production and proliferation. Increased telomere shortening is found in DS T-lymphocytes, which could lead to higher apoptosis rates [13-16]. Recent studies however did not find increased apoptosis in peripheral T-lymphocytes, despite increased apoptosis markers on T-lymphocytes in earlier reports [10, 17, 18].

Functional impairment in DS T-lymphocytes is supported by decreased proliferative and antigen T-cell responses [10]. Functional T-lymphocyte impairment could explain the increased incidence of haematological malignancies.

B-lymphocytes

With ageing, fewer B-lymphocytes are produced in the bone marrow. Total peripheral B-lymphocyte numbers do not decline with age, but the composition of the peripheral B-lymphocyte compartment changes: antigen-experienced memory B-lymphocytes increase and naive B-lymphocytes decrease in number. Memory B-lymphocytes with a decreased susceptibility to apoptosis accumulate in elderly persons, leading to clonal expansions of certain B-lymphocyte specificities, which may limit the diversity of the repertoire [3, 4].

Alterations of the B-lymphocyte compartment in DS are on the contrary present from birth onwards: newborns show decreased naive B-lymphocyte numbers, early expansion is absent, and memory B-lymphocytes do not increase with age. This results in extremely low total B-lymphocyte counts [19, 20].

Although serum immunoglobulin levels remain stable during normal ageing, antibodies generated in old age are of lower affinity because of an age-associated decrease in somatic hypermutation due to decreasing help from Th, and restricted B-lymphocyte repertoire due to clonal expansions.

In DS, despite the low B-lymphocyte numbers, a profound hypergammaglobulinemia develops from around 3 years of age onwards, without evidence of mono- or oligoclonality [21]. This hypergammaglobulinemia with increased IgG, IgG_1 and IgG_3, but decreased IgM, IgG_2 and IgG_4 serum levels [21] is more suggestive of dysregulation in class-switching and somatic hypermutation of B-lymphocytes within the germinal centers than an impaired B-lymphocyte production in the bone marrow. An altered Ig-pattern can result from both an intrinsic defect in DS B-lymphocyte activation and

proliferation or B-T-miscommunication in the periphery. Decreased numbers of Th type 2 – essential for the humoral immune response – have been reported in patients with DS [10] and could be associated with this altered Ig-pattern through impaired SHM and class-switching.

Immunizations

An indirect way to look at B-T-lymphocyte communication and the functional capacity of B- and T-lymphocytes is by studying vaccination responses. The specific antibody response to T-cell dependent protein antigens requires combined T-lymphocyte function, T-B interaction and B-lymphocyte function, whereas the response to T-cell independent polysaccharide antigens is largely determined by B-lymphocyte function alone.

The age-related decreased output and functional deficiency of the T- and B-lymphocyte pool hampers the adaptive immune response to both T-cell dependent and independent booster- and neo-vaccinations in the elderly [4]. Quantitative antibody responses are lower, decline faster and the affinity of the antibodies is diminished [4, 22-24].

Impaired specific antibody responses to both T-cell-dependent (e.g. tetanus [25], influenza [26]) and T-cell-independent (e.g. pneumococcal polysaccharide [27]) vaccines [28] are repeatedly reported in DS as well, which suggests both altered T- and B-lymphocyte function and communication.

Clinical relevance

The restricted B- and T-lymphocyte repertoire in the elderly, with antibodies of lower affinity, leads to immunodeficiency and immunodysregulation, resulting in a trias of higher infection and malignancy rates and more auto-immune phenomena such as rheumatoid arthritis (RA) [29]. This clinical trias is seen in DS as well. The increased susceptibility to respiratory tract infections in DS could – at least in part – be explained by a combination of anatomic and functional ear-nose-throat abnormalities, hypotonia, mental retardation and increased incidence of gastro-oesophageal reflux, but these cannot explain the increased auto-immune phenomena and hematological malignancies [30]. Although patients with DS have the same clinical trias of increased infections, malignancies and auto-immune diseases as the elderly, in practice the actual pathogens and disease-burden are to some extent different [31]. In DS a higher frequency of mainly hypothyroidism [33], celiac disease [34] and diabetes mellitus type 1 [35] is seen. Malignancies in DS consist mostly of haematological ones in contrast to elderly with an increase in non-haematological malignancies as well [36].

Progeria syndromes

Progeria syndromes (PS) are very rare diseases, leading to precocious and/or accelerated ageing [7]. PS form a clinically and genetically heterogenous group. Most

PS are only segmental in nature; they do not cause early or accelerated ageing in all human cell lines. Also, humans with PS can present symptoms that are not common during normal ageing [7]. PS can be further subdivided in different groups on the basis of the underlying genetic defect [7]. Hutchinson-Gilford progeria syndrome (HGPS) is the first described PS; LMNA gene mutation results in accumulation of lamin A protein in the cell nucleus. Werner syndrome – also called adult-onset progeria – is caused by a mutation in ATP dependent helicase leading to repair defects in DNA-double-strand breaks, but with slower onset than HGPS. Cockayne syndrome (CS) and xeroderma pigmentosum (XP) are examples of DNA-repair defect disorders causing progeria-like disease. Because of the segmental nature of most PS, it is not always clear whether and to what extent haematological cell lines are influenced [7]. The immune system in these progerias has only been studied in case reports and small cohort studies. In a case report of a child with HGPS [37] increased B-lymphocyte numbers with severely decreased IgG and IgA serum levels were seen. In 12 adults with HGPS however normal levels of Igs and normal B- and T-lymphocyte counts were found (including Th and Tc) [38]. Five adults with Werner syndrome showed normal immunoglobulin levels, normal relative B- and normal to decreased relative T-lymphocyte counts [39]. Neoplasms and infections were found to be more frequent in Werner syndrome, which could be interpreted as clinically suggestive of immuno-senescence. However, these neoplasms (eg sarcomas, melanomas) do not always overlap with commonly occurring neoplasms in ageing [40].

XP patients have normal T-lymphocyte (including Tc and Th) counts, but decreased natural killer activity [41-43]. CS patients show normal T-lymphocyte counts; their NK-activity is normal [41]. XP patients have an increased risk of skin cancer, but apparently no increased risk of infections and auto-immune diseases. CS patients have no associated increased cancer risk, possibly due to normal NK cell and T-lymphocyte related immune surveillance [41].

More research is needed, but it seems that the picture in progeria and progeria-like syndromes is not comparable to normal 'immunosenescence'; it is not the same in the different subtypes of PS and moreover is very different from the picture in DS.

CONCLUSION

At first sight, the DS profile seems to fit in with precocious immunosenescence, as thymic involution with low thymic output and T-lymphocyte dysregulation resulting in higher rates of infections, malignancies and auto-immune disease occur both in DS and normal ageing. Appearances however can be deceptive.

The decreased naive B- and T-lymphocyte production from birth onwards combined with the lack of compensatory memory cell expansion and proliferation do

not support precocious immunosenescence in DS; these findings fit intrinsic immunodeficiency better. The hypergammaglobulinemia in combination with decreased specific antibody responses support the theory that patients with DS harbor a combined T- and B-lymphocyte immunodeficiency with different mechanisms involved than in normal ageing. Progeria syndromes are not comparable to DS either. PS show segmental aspects of accelerated/precocious ageing at most reflected in minor lymphocyte changes. Also, the PS clinical profile differs from DS with respect to the occurrence of malignancies, infections and auto-immune diseases.

Therefore, we challenge the hypothesis that the immunological abnormalities in Down syndrome should be interpreted as precocious immunosenesence. More genetic and immunological research is needed to study the true nature of the effect of an extra chromosome 21 on the immune system in DS.

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4

BOTH NORMAL MEMORY COUNTS AND DECREASED NAIVE CELLS FAVOR INTRINSIC DEFECT OVER EARLY SENESCENCE OF DOWN SYNDROME T-LYMPHOCYTES

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ABSTRACT

Because of their increased malignancies, autoimmune diseases, and infections, patients with Down syndrome (DS) show features of immunodeficiency. The DS thymus and T-lymphocyte subsets have indeed proven to be different, and this has been interpreted as precocious ageing. Our study on T-lymphocyte subpopulations in DS shows that the normal expansion of naive helper- ($CD4^+CD45RA^+$) and cytotoxic ($CD8^+CD45RA^+CD27^+$) T-lymphocytes is lacking in the first years of life; which is more logically explained by an intrinsic T-lymphocyte defect. Furthermore, memory cell numbers are not different from age-matched controls (AMC), which do not support the hypothesis of precocious ageing. Although the absolute numbers of T-lymphocyte subpopulations approach AMC levels towards adulthood, the persistent clinical problems suggest that these cells may not function optimally. However, the clinical picture does not fit severe T-lymphocyte deficiency. The latter concept is also supported by our finding that cytomegalovirus (CMV)-seropositive DS children show similar numbers of terminally differentiated cytotoxic T-lymphocytes when compared to healthy children, instead of increased numbers as are seen in immunocompromised hosts.

INTRODUCTION

Down syndrome (DS) is associated with a high frequency of haematological malignancies [1-4], autoimmune diseases like celiac disease and hypothyroidism [5-7], as well as recurrent, mainly respiratory, infections [2, 8]. This fits with immunodeficiency. Indeed, the thymus in DS children is smaller and abnormal [9-13], and blood T-lymphocyte subpopulations differ from healthy controls [14-16]. This has been interpreted as precocious ageing of the immune system due to the lower relative number of $CD4^+CD45RA^+$ naive T-lymphocytes [17, 18] and lower T-cell-receptor excision circle counts [19, 20] in DS children. However, we recently showed [21] that the vast expansion of T-lymphocytes in the first years of life is abrogated, favoring an intrinsic defect. We studied T-lymphocyte subpopulations in DS children compared with age-matched controls (AMC) to analyze whether the results support this alternative theory.

T-lymphocyte differentiation and expansion are influenced by encountered viral infections. Especially, the expansion of $CD45RA^+CD27^-$ terminally differentiated cytotoxic T-lymphocytes (Tc), which is described as unique for cytomegalovirus (CMV) infection. The individual set-point is defined by the degree of immunocompetence during the primary CMV-contact: immunodeficient children show higher median absolute numbers of terminally differentiated Tc [22]. To further assess the degree of immuno(in)competence in DS, we related T-lymphocyte subpopulations to CMV-serostatus and compared the DS children with groups from the literature with different immune status during primary CMV-contact [22, 23].

METHODS

Study population

An extra 3 ml of EDTA blood and 7 ml of blood without additive was drawn from 95 non-institutionalized DS children (49 males; mean age 7 years, range 1-20) visiting the Jeroen Bosch Hospital, 's-Hertogenbosch, or the Rijnstate Hospital, Arnhem, The Netherlands, during routine follow-up of thyroid function after parental informed consent. All children were otherwise healthy at the time of sampling. Leftover EDTA blood from 33 healthy AMC children who underwent venipuncture for e.g. pre-operative screening for minor surgery, was used as control. The study was approved by the local Medical Ethics Committees of all participating hospitals.

We divided the children into the same age groups that were used in a large Dutch reference study analyzing lymphocyte subpopulations [24]. Absolute and relative numbers of T-lymphocyte subpopulations were compared in DS and AMC children. Absolute numbers of terminally differentiated Tc of CMV-seropositive (CMV^+) DS

children were compared with CMV-seronegative (CMV^-) DS children and with the results from the evaluation and discussion from recent literature [22, 23] including children with human immunodeficiency (HIV) infection, children using immunosuppressive therapy and children who were otherwise healthy at the time of primary CMV-contact.

Immunophenotyping

Three-color flow cytometric immunophenotyping was performed to determine T-lymphocyte subpopulations in both DS and AMC using the lysed whole-blood method. FITC-, phycoerythrin (PE)- and PE-cyanin 5 (PE-Cy5)-conjugated MAb were used with the following antigen specificity: CD3 (PE-Cy5; Immunotech, Marseille, France), CD3/CD4 (FITC/PE; IQProducts, Groningen, The Netherlands), CD8 (PE-Cy5; Immunotech), CD14 (PE; Beckton Dickinson (BD), San Jose, CA, USA), CD15 (FITC; IQ Products), CD16/CD56 (FITC; BD), CD19 (PE-Cy5; Immunotech), CD27 (FITC; BD), CD45 (PE-Cy5; Immunotech), CD45RA (PE; Coulter Immunology, Hialeah, USA), CD45RO (FITC; Serotec DPC, Apeldoorn, The Netherlands), TCR-αβ (PE-Cy5;BD) and TCR-γδ (PE; BD).

Aliquots were incubated for 15 minutes at room temperature with different combinations of optimally titrated conjugated MAb to determine the following lymphocyte subpopulations: T-lymphocytes ($CD3^+$), B-lymphocytes ($CD19^+$), natural killer (NK) cells ($CD16^+$and/or$CD56^+CD3^-$), helper-T-lymphocytes (Th) ($CD3^+CD4^+$), Tc ($CD3^+CD8^+$), naive Th ($CD3^+CD4^+CD45RA^+$), memory Th ($CD3^+CD4^+CD45RO^+$), naive Tc ($CD8^+CD45RA^+CD27^+$), central memory Tc ($CD8^+CD45RA^-CD27^+$), effector memory Tc ($CD8^+CD45RA^-CD27^-$), terminally differentiated Tc ($CD8^+CD45RA^+CD27^-$), $CD3^+$TCR-$\alpha\beta^+$ and $CD3^+$TCR-$\gamma\delta^+$ T-lymphocytes. Erythrocytes were lysed using FACSLysing solution (BD) according to the manufacturer's protocol. The remaining cells were washed twice with PBS with BSA and analyzed by flow cytometry after calibration with the SPHERO CaliFlow kit (Spherotech, Libertyville, IL, USA) as recommended by the European Working Group on Clinical Cell Analysis [25]. A FACScan or FACSCalibur flow cytometer (BD) was used. Absolute leukocyte counts were determined with a Sysmex SE-9500 hematology analyzer (Sysmex, Kobe, Japan). The lymphocyte gate was checked with a CD15/CD14/CD45 triple labeling and considered correct if <5% contamination was present. B-lymphocytes and NK cells were used to check whether the T+B+NK equaled 100 ± 5%. Absolute numbers of lymphocyte subpopulations were calculated by multiplying the absolute leukocyte count ($x10^9$/l) by the relative total lymphocyte size (%) and relative size of the lymphocyte subpopulation (%).

CMV serology and PCR

Anti-CMV IgM and IgG were measured in duplo by enzyme-linked fluorescence analysis using the VIDAS test in 90 DS children (Biomerieux, Lyon, France); for 5 DS

children, serum was not available. IgG avidity tests (VIDAS, Biomerieux) were performed to differentiate between recent (<3 months) and late CMV contact. Real-time PCR for CMV-DNA [22] was performed in CMV-IgG$^+$ children to differentiate between active and latent infection.

Review of medical files

The medical files of 91/95 DS children were reviewed retrospectively; four files were unavailable. The 91 children were divided into four groups: 1) no increased infection rate, 2) increased infection rate (age at inclusion <8 years), 3) increased infection rate (age at inclusion >8 years), and 4) increased infection rate until, but not after the age of 8 years. In addition, the presence of celiac disease or autoimmune hypothyroidism was noted.

Statistical analysis

To compare the T-lymphocyte subpopulations between DS and AMC the Mann Whitney U-test was used ($p<0.05$). An analysis of variance (completely randomized two-factorial design; $p<0.05$) was applied to the data to test the overall effects of age (2-16 years) and group (DS and AMC). Age groups with low numbers of AMC children were excluded (<2 yr; >16 yr) from this analysis. Levene's test for equality of error variances was used on all subpopulations ($p<0.05$). Tc subpopulations of CMV-sero-negative (CMV$^-$) and CMV$^+$ DS children were analyzed after log transformation by *t* test ($p<0.05$). All analyses were performed with SPSS 16.0 for Windows.

RESULTS

The absolute and relative numbers of the analyzed CD3$^+$ T-lymphocyte subpopulations, and the results of the statistical analyses are listed in table 1 (the values for CD3$^+$ T-lymphocytes, CD3$^+$CD4$^+$ Th, and CD3$^+$CD8$^+$ Tc were reported before [21]). None of the interaction effects were significant. This means that the effects of age on the various T-lymphocyte subpopulations do not differ between DS children and AMC children; although T-lymphocytes and Th lack the expansion normally seen in the first years of life [24], the overall pattern seen in time is the same in DS and AMC. Clinically relevant data is presented in Table 2. We did not find a relation between any of the determined T-lymphocyte subpopulations and the incidence of infections or autoimmune diseases in these DS children.

Naive and memory CD3$^+$CD4$^+$ Th

The absolute numbers of CD45RA$^+$ naive Th are reduced in DS children. Naive Th lack the expansion seen in AMC children during the first years of life, but the overall pattern

Table 1 Absolute and relative numbers of T-lymphocyte subpopulations.

		9–15 months		15–24 months		2–5 years		
T-lymphocytes	**DS**	**1.94** (0.98-4.29)	n=11	**2.10** (1.22-2.79)	n=8	**1.54** (0.81-3.07)	n=16	
		69 (58-81)	n=11	**73** (66-81)	n=8	**74** (65-84)	n=16	
	AMC	5.00	n=1	2.30	n=1	1.95 (1.20-2.90)	n=10	
		70	n=1	60	n=1	66 (59-71)	n=10	
Th	**DS**	**1.22** (0.69-2.97)		**1.36** (0.70-1.59)		**0.78** (0.04-1.67)		
		69 (45-79)		**62** (47-78)		**55** (41-71)		
	AMC	3.30		1.40		1.15 (0.70-2.00)		
		65		60		60 (52-69)		
Tc	**DS**	**0.67** (0.20-1.20)		**0.76** (0.38-1.32)		**0.54** (0.28-1.41)		
		30 (20-41)		**36** (19-50)		**41** (26-58)		
	AMC	1.40		0.80		0.70 (0.50-1.10)		
		29		33		35 (29-47)		
Th naive	**DS**	**0.91** (0.50-1.90)		**0.90** (0.39-1.12)		**0.49** (0.19-1.15)		
		73 (63-83)		**68** (55-72)		**58** (37-70)		
	AMC	2.80		1.16		0.82 (0.41-1.60)		
		86		83		70 (58-80)		
Th memory	**DS**	**0.22** (0.13-0.72)		**0.25** (0.16-0.35)		**0.27** (0.14-0.39)		
		18 (10-28)		**23** (16-33)		**32** (22-50)		
	AMC	0.36		0.17		0.29 (0.18-0.38)		
		11		12		23 (16-38)		

	5–10 years		10 –16 years		>16 years		DS vs AMC (2-16 years) *	Age effect (DS+AMC; 2-16 years) **
	1.39 (0.62-2.67)	n=38	**1.00** (0.54-2.45)	n=19	**1.25** (1.06-1.56)	n=3	p=0.003	p<0.001
	74 (58-89)	n=38	**69** (59-85)	n=19	**74** (72-81)	n=3	p<0.001	NS
	1.85 (1.50-2.40)	n=8	1.60 (0.80-2.40)	n=11	1.40 (1.10-1.70)	n=2		
	69 (58-72)	n=8	65 (59-77)	n=10	69 (63-75)	n=2		
	0.63 (0.30-1.40)		**0.59** (0.28-1.16)		**0.81** (0.48-0.92)		p<0.001	p<0.001
	46 (25-65)		**58** (31-65)		**52** (45-73)		p<0.001	NS
	1.00 (0.90-1.60)		0.80 (0.50-1.20)		0.95 (0.80-1.10)			
	65 (49-71)		56 (41-73)		70 (68-71)			
	0.70 (0.27-1.45)		**0.45** (0.18-1.15)		**0.51** (0.33-0.78)		p<0.001	NS
	47 (33-71)		**40** (29-59)		**48** (27-51)		p<0.001	NS
	0.50 (0.40-0.80)		0.50 (0.30-1.00)		0.40 (0.30-0.50)			
	29 (26-45)		36 (22-49)		29			
	0.28 (0.07-0.97)		**0.20** (0.01-0.43)		**0.19** (0.06-0.29)		p<0.001	p<0.001
	44 (11-69)		**37** (2-59)		**24** (7-60)		p<0.001	p<0.001
	0.73 (0.64-1.30		0.54 (0.27-0.80)		0.57 (0.44-0.70)			
	72 (64-80)		61 (54-67)		59 (55-64)			
	0.27 (0.12-0.51)		**0.25** (0.15-0.65)		**0.50** (0.15-0.66)		NS	NS
	45 (21-74)		**52** (30-70)		**62** (31-72)		p<0.001	p<0.001
	0.24 (0.14-0.39)		0.25 (0.12-0.44)		0.31 (0.27-0.43)			
	23 (15-30)		31 (28-39)		32 (31-34)			

Table 1 Continued.

		9–15 months	15–24 months	2–5 years	
Tc naive	**DS**	**0.45** (0.11-0.83)	**0.26** (0.14-0.63)	**0.29** (0.09-0.85)	
		72 (57-82)	**53** (19-82)	**47** (18-86)	
	AMC	0.64	0.69	0.53 (0.27-0.71)	
		46	86	66 (49-89)	
Tc centr mem	**DS**	**0.15** (0.04-0.34)	**0.13** (0.06-0.26)	**0.19** (0.06-0.91)	
		23 (15-41)	**23** (8-35)	**39** (11-65)	
	AMC	0.53	0.10	0.16 (0.08-0.32)	
		38	12	28 (11-33)	
Tc eff mem	**DS**	**0.01** (0.00-0.02)	**0.04** (0.00-0.54)	**0.06** (0.01-0.61)	
		1 (0-3)	**9** (0-45)	**4** (1-34)	
	AMC	0.17	0.01	0.01 (0.00-0.14)	
		12	1	2 (0-13)	
Tc term diff	**DS**	**0.01** (0.00-0.06)	**0.02** (0.00-0.21)	**0.01** (0.00-0.32)	
		2 (0-9)	**3** (0-18)	**1** (0-31)	
	AMC	0.05	0.01	0.00 (0.00-0.11)	
		3	1	2 (0-10)	
$CD3^+TCR\alpha\beta^+$	**DS**	**1.89** (0.91-3.75)	**1.94** (1.15-2.65)	**1.44** (0.66-2.69)	
		93 (77-98)	**95** (90-96)	**91** (68-97)	
	AMC	4.83	2.13	1.79 (1.07-2.76)	
		97	93	92 (86-97)	

	5–10 years	10 –16 years	>16 years	DS vs AMC (2-16 years) *	Age effect (DS+AMC; 2-16 years) **
	0.23 (0.03-0.62)	**0.16** (0.05-0.55)	**0.11** (0.08-0.15)	p=0.001	p<0.001
	42 (10-74)	**41** (13-69)	**31** (11-31)	p<0.001	NS
	0.34 (0.23-0.54)	0.25 (0.16-0.64)	0.23 (0.15-0.30)		
	68 (39-77)	57 (37-80)	54 (49-59)		
	0.23 (0.09-0.65)	**0.14** (0.07-0.50)	**0.23** (0.20-0.25)	p=0.012	NS
	37 (12-62)	**37** (20-60)	**50** (30-62)	p=0.001	NS
	0.13 (0.07-0.31)	0.12 (0.07-0.23)	0.13 (0.09-0.17)		
	26 (17-56)	32 (13-39)	32 (30-34)		
	0.09 (0.01-0.61)	**0.07** (0.01-0.68)	**0.09** (0.03-0.70)	p=0.001	NS
	8 (1-42)	**7** (2-48)	**10** (3-52)	p=0.008	NS
	0.02 (0.01-0.38)	0.02 (0.01-0.16)	0.03 (0.01-0.04)		
	4 (2-6)	7 (1-22)	9 (2-15)		
	0.03 (0.01-0.38)	**0.02** (0.00-0.21)	**0.04** (0.01-0.06)	NS	NS
	5 (1-41)	**8** (1-27)	**7** (3-8)	p=0.044	NS
	0.02 (0.00-0.06)	0.02 (0.00-0.09)	0.02 (0.02-0.03)		
	3 (1-10)	4 (2-10)	5 (5-5)		
	1.20 (0.57-2.49)	**0.91** (0.49-2.12)	**1.19** (0.87-1.36)	p=0.001	p=0.001
	89 (61-95)	**91** (74-98)	**88** (82-96)	NS	NS
	1.64 (1.38-2.28)	1.42 (0.74-2.17)	1.34 (1.05-1.63)		
	93 (84-98)	89 (80-96)	96 (96-96)		

Table 1 Continued.

		9–15 months		15–24 months		2–5 years		
$CD3^+TCR\gamma\delta^+$	**DS**	**0.17** (0.03-0.52)		**0.11** (0.05-0.18)		**0.13** (0.04-0.53)		
		7 (2-23)		**5** (3-10)		**9** (3-32)		
	AMC	0.16		0.17		0.14 0.07-0.33)		
		3		7		8 (3-14)		

Grey shaded areas: absolute numbers of T-lymphocyte subpopulations (10^9 cells/l), blank background: relative numbers (%); AMC = age-matched control children; DS = Down syndrome children; NS = not significant; centr mem = central memory; eff mem = effector memory; term diff = terminally differentiated
* Analysis of variance, effect of group (children aged 2-16 years).** Analysis of variance, DS and AMC children together (2 to 16 years), effect of age.

seen in time is the same in both DS children and AMC children. Despite increased relative numbers of $CD45RO^+$ memory Th, absolute numbers do not differ from AMC children due to the lower absolute counts of total Th in DS children. The memory Th subset does not show an age-related change in size in either DS or AMC children (Figure 1).

Naive, central memory, effector memory and terminally differentiated $CD8^+$ Tc

Like naive Th, the absolute numbers of $CD45RA^+CD27^+$ naive Tc are decreased in DS, and lack the normal expansion seen in AMC during the first years of life, although the overall pattern seen in time is the same in DS and AMC. The absolute numbers of both $CD45RA^-CD27^+$ central memory and $CD45RA^-CD27^-$ effector memory Tc are higher in DS than in AMC, but values differ widely (Levene's test: $p=0.009$ and $p=0.003$, respectively). The absolute numbers of $CD45RA^+CD27^-$ terminally differentiated Tc are low in all age groups in both DS as well as AMC children. Neither in memory Th, the memory Tc subsets show an age-related change in size in DS or AMC (Figure 2).

TCR-$\alpha\beta^+$ and TCR-$\gamma\delta^+$ $CD3^+$T-lymphocytes

The absolute number of TCR-$\gamma\delta^+$ T-lymphocytes in DS children is lower, but values widely differ in AMC children (Levene's test: $p=0.000$). As was expected, the absolute numbers of TCR-$\alpha\beta^+$ T-lymphocytes follow the pattern of total T-lymphocytes.

	5–10 years	10 –16 years	>16 years	DS vs AMC (2-16 years) *	Age effect (DS+AMC; 2-16 years) **
	0.14 (0.04-0.66)	**0.09** (0.02-0.33)	**0.18** (0.04-0.18)	p=0.043	NS
	11 (5-39)	**9** (2-26)	**12** (3-17)	NS	NS
	0.12 (0.03-3.24)	0.17 (0.06-1.48)	0.06 (0.05-0.06)		
	7 (2-16)	11 (4-19)	4 (4-5)		

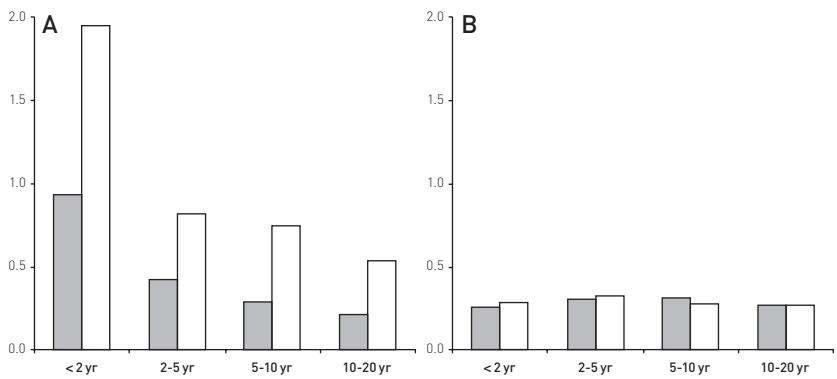

Figure 1 Median absolute numbers (x 10^9/l) of helper-T-lymphocytes per age group.

A. Naive helper-T-lymphocytes ($CD3^+CD4^+CD45RA^+$)
B. Memory helper-T-lymphocytes ($CD3^+CD4^+CD45RO^+$).

Grey bars: Down syndrome children; white bars: age-matched reference values.

CMV status and terminally differentiated CD8+ Tc

Twenty of the 90 tested DS children are CMV^+, they all have a latent infection (IgG^+IgM^-; PCR^- $Avidity^{hi}$). The median absolute number of $CD45RA^+CD27^-$ terminally differentiated Tc in CMV^+ DS children is 0.079 x 10^9 cells/l (range 0.007-0.36), and 0.017 x 10^9 cells/l (range 0.0004-0.22) in CMV^- DS children ($p<0.001$). In CMV^+ healthy children a median absolute number of $CD45RA^+CD27^-$ terminally differentiated Tc of 0.067 x 10^9 cells/l is described [22]. Higher absolute numbers are described in children with primary CMV infection during immunosuppressive therapy (median 0.413 x 10^9 cells/l) and in CMV^+ HIV-1 infected children (median 0.369 x 10^9 cells/l) (22, 23] (Figure 3).

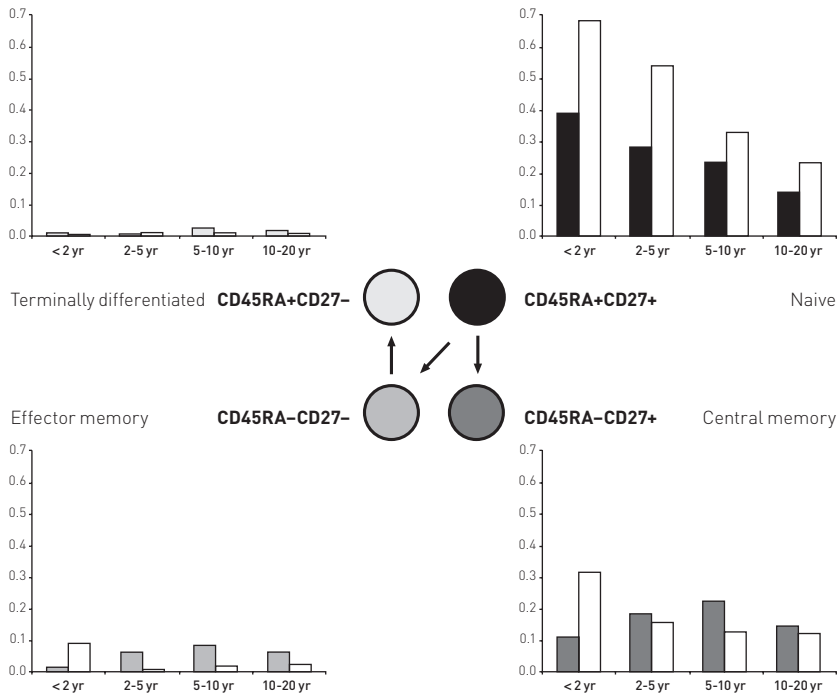

Figure 2 Median absolute numbers (x 10^9/l) of cytotoxic T-lymphocyte subpopulations per age group.

Grey and black bars: Down syndrome children; white bars: corresponding age-matched reference values.

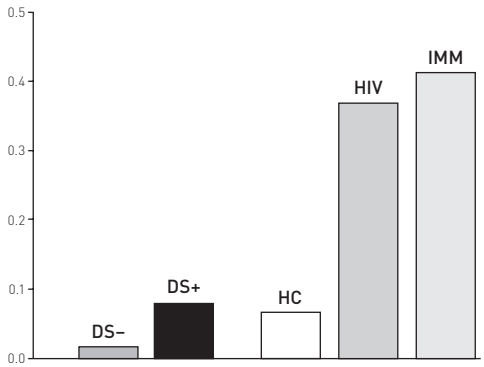

Figure 3 Median absolute numbers (x 10^9/l) of terminally differentiated cytotoxic T-lymphocytes per group.

CMV: cytomegalovirus; DS: Down syndrome; HC: healthy children; HIV: human immunodeficiency virus infected; IMM: receiving immunosuppressive medication during primary cytomegalovirus infection.

DISCUSSION

DS children lack the expansion of both naive Tc and naive Th normally seen in the first years of life; memory Tc and memory Th are not influenced by age in either DS or AMC children. With advancing age, numbers of memory Th, terminally differentiated Tc and TCR-γδ$^+$ T-lymphocytes normally increase [17]. Despite earlier statements in the literature suggesting precocious ageing of T-lymphocytes in DS, no early shift towards these T-lymphocyte subsets occurred in our DS cohort. A more likely explanation of the observed T-lymphocyte subset alterations in DS children would therefore be that the decreased numbers of Tc but particularly of Th are the result of (partial) failure of T-lymphocyte generation, an intrinsic T-lymphocyte defect, increased apoptosis or a combination of these.

It is interesting to speculate about this finding. Apoptosis data in DS is scarce, but Elsayed [26] recently described increased early apoptosis markers in DS T-lymphocytes. Thymic alterations in DS are well-known [9, 10, 12, 13], and are already described in DS fetuses [27], suggesting T-lymphocyte generation is impaired by a defect in the DS thymus. This situation resembles children with DiGeorge syndrome who have a smaller or absent thymus; they demonstrate decreased (total) Th and Tc subsets as well [28, 29]. In DiGeorge syndrome, however, most cases appear to gradually reach T-lymphocyte levels of healthy adults over time. In comparison, naive Tc in our DS children reach normal levels during adolescence, but naive Th remain decreased. It is

still uncertain whether these cells function normally, having shown such a profound lack of the antigen-driven expansion in earlier years. In vitro tests of T-lymphocyte function support this hypothesis of impaired functioning [30].

Clinically speaking, DS children do not show signs of a profound T-lymphocyte deficiency. This corresponds with our finding that CMV^+ DS children show absolute numbers of terminally differentiated Tc comparable to healthy children, not to immunocompromised, e.g. HIV^+, children.

In conclusion, the observed T-lymphocyte alterations in DS are more likely caused by an intrinsic defect than by early senescence of the immune system. In the future, functional studies of T-lymphocytes may help to differentiate between a defect primarily originating in the thymus (as in DiGeorge syndrome), a defect in the T-lymphocytes themselves, increased apoptosis, or a combination of these options.

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DOWN SYNDROME B-LYMPHOCYTE SUBPOPULATIONS, INTRINSIC DEFECT OR DECREASED T-LYMPHOCYTE HELP?

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Published in Pediatric Research

ABSTRACT

Down Syndrome (DS) is known for increased incidence of respiratory infections and autoimmune diseases, indicating impaired immunity. Until now, attention has mainly been focused on T-lymphocytes. Therefore, we determined B-lymphocyte subpopulations in 95 DS children compared to 33 healthy age-matched control (AMC) children. DS serum immunoglobulin levels were compared with 962 non-DS children with recurrent infections. The results were combined with clinical data. Transitional and naive B-lymphocytes are profoundly decreased in children with DS. This could be caused by an intrinsic B-lymphocyte defect resulting in (partial) failure of B-lymphocyte generation, decreased antigen-induced proliferation and/or increased apoptosis, or by decreased proliferation due to deficient T-lymphocyte help, or a combination of these. The decreased $CD27^{+}$, $CD21^{high}$ and $CD23^{+}$ cells are reminiscent of common variable immunodeficiency and suggestive of disturbed peripheral B-lymphocyte maturation. Immunoglobulin levels in DS are abnormal – as has been described before – and different from non-DS children with recurrent infections. We conclude that the humoral immune system is abnormal in DS, but could not find a relation between B-lymphocyte subset counts, immunoglobulin levels and clinical features of the DS children in our cohort, nor could we answer the question whether DS B-lymphocytes are truly intrinsically deficient, or that all findings could be explained by deficient T-lymphocyte help.

INTRODUCTION

Down syndrome (DS) is associated with recurrent – mainly respiratory – infections [1, 2], decreased responses to vaccination [3-8], a higher frequency of hepatitis B surface antigen carriers [3] and autoimmune diseases like celiac disease and hypothyroidism [9-11]. These features are suggestive of immunodeficiency. Until now, attention has mainly been focused on the thymic alterations and decreased absolute numbers of T-lymphocytes in peripheral blood [12, 13]. We recently showed that a striking B-lymphocytopenia is present from the very beginning in patients with DS [14]. This B-lymphocytopenia could be due to an intrinsic B-lymphocyte defect, a deficient T-lymphocyte help, or a combination of these. An intrinsic B-lymphocyte defect could be due to (partial) failure of B-lymphocyte generation, decreased antigen-induced proliferation, and/or increased apoptosis. Deficient T-lymphocyte-help could lead to disturbed B-lymphocyte activation and proliferation. Despite the B-lymphocytopenia, a considerable hypergammaglobulinemia of IgA and IgG after the age of five years, with high levels of IgG_1 and IgG_3 and low levels of IgG_2 and IgG_4, is described [3, 15, 16].

This combination of profound B-lymhocytopenia and hypergammaglobulinemia favors a disturbance in T-lymphocyte help, with the possibility that immunoglobulins are oligoclonal in DS, and specific T-cell-dependent antibody responses inadequate. The latter has indeed been described [3, 15]. However, the T-cell-independent antibody response to pneumococcal polysaccharide antigen is also decreased in DS [4], suggesting an intrinsic B-lymphocyte defect is also present. We studied B-lymphocyte subpopulations in relation to relevant clinical features in 95 DS children, to further unravel this question.

METHODS

Study population

From 95 non-institutionalized children with DS (49 boys; Figure 1), either visiting the Jeroen Bosch Hospital, 's-Hertogenbosch, or the Rijnstate Hospital, Arnhem, The Netherlands, an extra 3ml of EDTA and 7ml of blood without additive was drawn during routine follow-up of thyroid function after parental informed consent. All children were otherwise healthy at the time of sampling. Left-over EDTA blood from 33 healthy age-matched control (AMC) children who underwent venipuncture, e.g. pre-operative screening for minor surgery, was used as control.

We retrospectively collected the titers of serum IgG, IgA and IgM that were determined for diagnostic purposes in 962 non-DS patients suffering from recurrent infections (younger than 21 years) between January 2006 and July 2008 in the Jeroen Bosch Hospital, 's-Hertogenbosch, and the Bernhoven Hospital, Oss/Veghel,

The Netherlands. In 285 of the 962 patients IgG-subclasses were also determined. The study was approved by the local Medical Ethics Committees of both hospitals.

Immunophenotyping

Three-color flowcytometric immunophenotyping was performed to determine B-lymphocyte subpopulations in both DS and AMC children using the lysed whole-blood method. FITC, phycoerythrin (PE), and PE-cyanin 5 (PE-Cy5) conjugated antibodies were used with the following antigen specificity: CD3 (PE-Cy5; Immunotech, Marseille, France), CD5 (FITC; Becton Dickinson (BD), San Jose, CA, USA), CD10 (FITC; BD), CD16/CD56 (FITC; BD), CD14 (PE; BD), CD15 (FITC; IQProducts, Groningen, The Netherlands), CD19 (PE-Cy5; Immunotech), CD20 (PE; BD), CD21 (PE; BD), CD23 (PE; BD), CD27 (FITC; BD), CD38 (PE; BD), CD45 (PE-Cy5; Immunotech), κ (PE; Dako, Carpinteria, CA, USA), and λ (PE; Dako). In all children T-lymphocytes ($CD3^+$), B-lymphocytes ($CD19^+$), natural killer (NK) cells ($CD16^+$and/or$CD56^+CD3^-$) and CD21

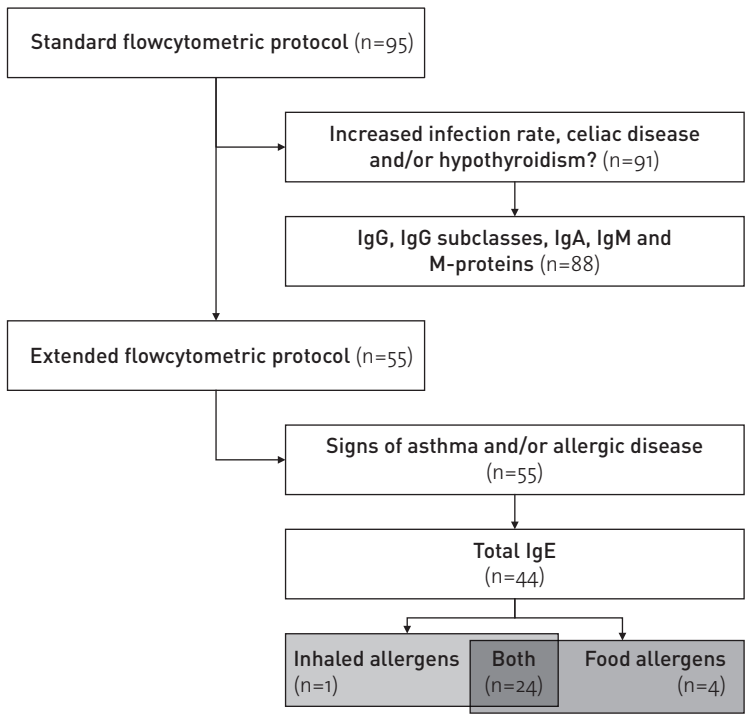

Figure 1 Patient flow diagram.

Flow diagram showing group size of patients with Down syndrome in relation to assessed variables.

and CD5 expression on $CD19^+$ B-lymphocytes were determined. An extended protocol was used in the last included 55 children. In this group CD10, κ and λ expression, CD27 and CD20 expression, CD27 and CD38 expression, and CD27 and CD23 expression on $CD19^+$ B-lymphocytes were also analyzed.

Aliquots were incubated for 15 minutes at room temperature in the dark with different combinations of optimally titrated antibodies. Only for the samples that were incubated with anti-κ or anti-λ antibodies, the aliquots were washed three times with 0.5% BSA/PBS before incubation. Erythrocytes were lysed using FACSLysing solution (BD) according to the manufacturer's protocol. The remaining cells were washed twice with BSA/PBS and analyzed by flow cytometry after calibration with the SPHERO CaliFlow kit (Spherotech, Libertyville, IL, USA) as recommended by the European Society for Clinical Cell Analysis [17]. A FACScan or FACSCalibur flow cytometer (BD) was used in combination with CellQuest or CellQuest Pro software (BD). The lymphocyte-gate was checked with a CD15/CD14/CD45 triple labeling and considered correct if <5% contamination was present. T-lymphocytes and NK cells were used to check whether the 'lymphosum' (B+T+NK) equaled 100 ± 5%. Absolute leukocyte counts were determined with a Sysmex SE-9500 hematology analyzer (Sysmex, Kobe, Japan). Absolute numbers of B-lymphocyte subpopulations were calculated by multiplying the absolute leukocyte count ($x10^9$/l) by the relative total lymphocyte size (%) and relative size of the lymphocyte subpopulation (%).

Immunoglobulins

For 88 of the 95 children with DS, serum IgG, IgG_1, IgG_2, IgG_3, IgG_4, IgA, and IgM were studied; in seven children, serum was not available. IgG, IgA and IgM were determined by kinetic nephelometry (Beckman Coulter Array 360, Beckman Coulter, Fullerton, CA, USA); IgG-subclasses were assessed by kinetic nephelometry using a human IgG-subclass nephelometry kit (Sanquin Reagents, Amsterdam, The Netherlands).

Qualitative M-proteins were assessed by serum electrophoresis on alkaline buffered (pH 9.2) agarose gels by a Hydrasys system (Sebia, GA, USA). In cases of uncertainty, additional serum electrophoresis using immunofixation with monovalent antiserum was performed.

IgE was measured in 44 of the 55 children included in the extended protocol using a sandwich chemiluminescent immunoassay (Immulite 2500, DPC/Siemens, IL, USA); the volume of serum available was insufficient in 11 children. Specific IgE testing (Immulite 2500) of food and inhaled allergens was performed in 28 and 25 children, respectively. When insufficient serum was available, we tested for food allergens only for children aged <2 years and inhaled allergens only for children aged >2 years. The Fp5 food allergen panel (DPC/Siemens) contained egg white, cow's milk, codfish, soya, peanut and wheat allergen. The AlaTOP inhaled allergen panel (DPC/Siemens),

contained house mite (*Dermatophagoides pteronyssimus*), cat dander epithelium, dog dander, Bermuda grass, timothy grass, *Penicillium notatum*, *Alternaria tenuis*, birch, Japanese cedar, common ragweed (*Ambrosia artemisiifolia*), and English plantain and *Parietaria officinalis* allergen. To interpret the IgE results, we used our laboratory cut-off values of <50 U/ml for children aged <10 years, and <90 U/ml for children aged >10 years and adults.

Review of medical files

The medical files of 91 of the 95 children with DS were reviewed retrospectively; four files were unavailable. The 91 children were divided into four groups: 1) no increased infection rate, 2) increased infection rate (age at inclusion <8 years), 3) increased infection rate (age at inclusion >8 years), and 4) increased infection rate until, but not after, the age of 8 years. The presence of celiac disease or autoimmune hypothyroidism was noted. In addition, the 55 patients of the extended protocol were also divided into positive or negative for symptoms of asthma and/or allergic disease (recurrent cough, persistent wheeze, admission on a pediatric ward for asthma exacerbation, clinical response to bronchodilators and/or inhalation corticosteroids, clinical signs of allergic disease).

Statistical analysis

An analysis of variance (completely randomized two-factorial design; $p<0.05$) was applied to the data. For this analysis, we excluded those age groups for which the number of AMC data was too low. The two fixed factors in the analysis of variance were age (three age groups: 2-5 years, 5-10 years, and 10-16 years) and the difference between DS and AMC children. Levene's test for equality of error variances was used, and the results are mentioned in the text only when $p<0.05$. The one sample *t* test ($p<0.05$) was used to compare the Ig values of DS children and non-DS children suffering from recurrent infections with the mean of age-matched reference values and each other [18, 19]. All analyses were performed with SPSS 16.0 for Windows.

RESULTS

B-lymphocyte subpopulations

The absolute and relative numbers of $CD19^+$ B-lymphocyte subpopulations with results of statistical analyses can be found in Table 1, clinically relevant data are presented in Table 2. We did not find a relation between any of the determined B-lymphocyte subpopulations and the incidence of infections or of allergic complaints and/or asthma in these children with DS. The values for $CD19^+$ B-lymphocytes were reported before [14]: the $CD19^+$ B-lymphocyte count is significantly decreased in all age groups in DS compared with AMC, and the enormous expansion, which is found in

healthy children in the first years of life, is lacking ([20]). The effect of age on the $CD19^+$ B-lymphocyte count is significantly different between DS and AMC children (interaction for absolute values; Table 1), so this finding is highly significant. The κ/λ ratio is slightly increased in older DS children. $CD5^+$ and $CD5^-$ B-lymphocytes follow the pattern of total $CD19^+$ B-lymphocytes in children with DS. There is no evident increase of "immature" B-lymphocytes in DS: $CD10^+$ and $CD20^-$ B-lymphocytes do not clearly differ between DS and AMC children.

$CD27^-CD38^{dim}$ naive B-lymphocytes and $CD27^-CD38^+$ transitional B-lymphocytes follow the pattern of total $CD19^+$ B-lymphocytes in DS as well. Unfortunately, we cannot differentiate between $CD27^+CD38^{dim}IgD^+$ marginal zone and $CD27^+CD38^{dim}IgD^-$ memory B-lymphocytes because the expression of IgD was not determined. Absolute and relative numbers of total $CD27^+$ B-lymphocytes are decreased in DS compared with AMC children; the absolute numbers show a slight increase during the $CD19^+$ B-lymphocyte expansion in the first years of life, which is less prominent in DS than in AMC children. The $CD27^+CD38^{++}$ plasma cell population is small in peripheral blood, but – unexpectedly – not different between DS and AMC children. The relative and absolute numbers of B-lymphocytes with high expression of CD21 ($CD21^{high}$) are significantly decreased in DS children; the absolute numbers decline with age, but more so in AMC than in DS children due to a higher initial peak in the former. The same holds true for CD23. The relative expression of CD23 within the $CD19^+$ B-lymphocyte population shows a far wider range in DS than in AMC children.

Immunoglobulins

The serum levels of IgG, IgG_1, IgG_2, IgG_3, IgG_4, IgA, and IgM found in the children with DS and in non-DS children with increased infection rates (see Methods section) in comparison with age-matched reference values are shown in Figure 2 [18,19]. In the DS group, mean IgG and IgG_1 are already higher than the age-matched reference values from the ages of 2 and 3 years onwards, respectively (one-sample t-test; $p<0.05$). Mean IgA and IgG_3 are normally distributed, but mean IgM and IgG_2 are lower in children with DS in all age groups. IgG_4 values are consistently very low in DS children. Mean Ig serum levels in the non-DS children with increased infection rates are similar to the children with DS for IgA and IgG_2, but mean IgG is higher in children with DS in some of the older age groups, and mean IgG_1 and IgG_3 are higher in children with DS from the ages of 3 and 2 years onwards, respectively. Mean IgM and IgG_4 are lower in children with DS than in the non-DS children with increased infection rates in the older age groups. We did not find any mono- or oligoclonal M-proteins in the 88 DS children tested. IgE is increased in six of the 44 DS children tested; five showed high relative percentages of $CD23^+$ B-lymphocytes which are within the range of the AMC group (one with asthma; data not shown). Specific IgE testing of food and inhaled allergens is negative in all children tested.

Table 1 Absolute and relative numbers of B-lymphocyte subpopulations in Down syndrome children compared to age matched controls.

		9 – 15 months		15 – 24 months		2 – 5 years		
Total CD19⁺ B-lymphocytes	**DS**	**0.46** (0.19-1.14)		**0.33** (0.16-0.76)		**0.28** (0.13-0.55)		
		18 (9-26)	n=11	**14** (8-24)	n=8	**13** (6-24)	n=16	
	AMC	1.47		0.91		0.75 (0.33-0.96)		
		20	n=1	33	n=1	23 (18-34)	n=10	
CD19⁺CD5⁺	**DS**	**0.22** (0.06-0.88)		**0.14** (0.04-0.30)		**0.08** (0.04-0.29)		
		49 (31-74)	n=11	**37** (23-55)	n=8	**30** (20-76)	n=16	
	AMC	0.64		0.40		0.26 (0.08-0.55)		
		43	n=1	43	n=1	36 (15-59)	n=9	
CD19⁺CD10⁺	**DS**	**0.02** (0.01-0.07)		**0.01** (0.01-0.06)		**0.02** (0.00-0.07)		
		0.5 (0.2-2.3)	n=7	**0.4** (0.2-3.4)	n=6	**0.5** (0.1-3.5)	n=7	
	AMC	0.03		0.03		0.01 (0.00-0.02)		
		0.4	n=1	0.9	n=1	0.3 (0.1-0.6)	n=10	
CD19⁺CD20⁻	**DS**	**0.00** (0.00-0.00)		**0.00** (0.00-0.01)		**0.00** (0.00-0.01)		
		5 (1-8)	n=8	**6** (3-18)	n=6	**5** (1-12)	n=7	
	AMC	0.00		0.00		0.00 (0.00-0.01)		
		2	n=1	2	n=1	2 (1-4)	n=10	
CD19⁺CD21⁻	**DS**	**0.04** (0.01-0.08)		**0.03** (0.01-0.06)		**0.03** (0.01-0.10)		
		7 (1-16)	n=11	**5** (3-26)	n=8	**9** (4-22)	n=16	
	AMC	0.04		0.00		0.05 (0.01-0.08)		
		3	n=1	0	n=1	6 (2-10)	n=9	

	5 – 10 years		10 – 16 years		>16 years		DS vs AMC*	Age effect for DS + AMC**	Interaction DS and AMC†
	0.19 (0.06-0.45)		**0.11** (0.05-0.58)		**0.11** (0.04-0.12)		p<0.001	p<0.001	p=0.001
	9 (5-22)	n=38	**8** (4-20)	n=19	**6** (3-7)	n=3	p<0.001	p<0.001	NS
	0.45 (0.32-0.66)		0.32 (0.19-0.60)		0.26 (0.23-0.29)				
	17 (12-21)	n=8	16 (11-21)	n=11	14 (10-17)	n=2			
	0.06 (0.01-0.15)		**0.03** (0.02-0.22)		**0.02** (0.01-0.02)		p<0.001	p<0.001	p=0.003
	29 (9-46)	n=38	**27** (16-47)	n=19	**21** (19-24)	n=3	NS	p=0.007	NS
	0.13 (0.1-0.22)		0.08 (0.02-0.28)		0.05 (0.04-0.06)				
	29 (21-38)	n=8	24 (7-46)	n=11	20 (13-27)	n=2			
	0.01 (0.00-0.02)		**0.00** (0.00-0.02)		**0.01** (0.00-0.01)		NS	p=0.003	p=0.031
	0.2 (0.04-1.4)	n=19	**0.1** (0.06-1.1)	n=13	**0.3** (0.04-0.5)	n=2	NS	NS	p=0.048
	0.01 (0.00-0.04)		0.01 (0.00-0.02)		0.01 (0.00-0.01)				
	0.3 (0.09-1.6)	n=8	0.3 (0.03-0.7)	n=11	0.3 (0.2-0.4)	n=2			
	0.00 (0.00-0.01)		**0.00** (0.00-0.00)		**0.00** (0.00-0.00)		NS	NS	NS
	4 (2-20)	n=19	**3** (1-9)	n=13	**2** (2-2)	n=2	p<0.001	NS	NS
	0.00 (0.00-0.00)		0.00 (0.00-0.01)		0.00 (0.00-0.00)				
	2 (1-3)	n=7	1 (0-8)	n=11	4 (3-5)	n=2			
	0.02 (0.00-0.17)		**0.01** (0.00-0.05)		**0.00** (0.00-0.02)		NS	p<0.001	NS
	11 (2-39)	n=38	**9** (4-20)	n=19	**6** (3-14)	n=3	p<0.001	NS	NS
	0.02 (0.00-0.04)		0.01 (0.00-0.02)		0.02 (0.01-0.02)				
	3 (1-8)	n=8	2 (1-4)	n=11	5 (5-5)	n=2			

Table 1 Continued.

		9 – 15 months		15 – 24 months		2 – 5 years		
$CD19^+CD21^{low}$	**DS**	**0.09** (0.03-0.19)		**0.07** (0.04-0.17)		**0.03** (0.02-0.09)		
		22 (6-41)	n=11	**20** (10-29)	n=8	**14** (6-24)	n=16	
	AMC	0.22		0.06		0.06 (0.01-0.08)		
		15	n=1	6	n=1	8 (3-18)	n=9	
$CD19^+CD21^{high}$	**DS**	**0.25** (0.15-0.98)		**0.25** (0.09-0.65)		**0.22** (0.11-0.43)		
		69 (48-93)	n=11	**70** (52-86)	n=8	**77** (62-84)	n=16	
	AMC	1.22		0.87		0.68 (0.25-0.76)		
		82	n=1	94	n=1	86 (72-95	n=9	
$CD19^+CD23^+$	**DS**	**0.36** (0.08-0.56)		**0.23** (0.07-0.53)		**0.24** (0.08-0.37)		
		66 (28-80)	n=8	**73** (26-88)	n=6	**61** (47-98)	n=7	
	AMC	1.38		0.60		0.62 (0.29-0.93)		
		92	n=1	64	n=1	82 (66-98)	n=10	
$CD19^+CD27^+$	**DS**	**0.04** (0.03-0.06)		**0.04** (0.03-0.06)		**0.05** (0.02-0.08)		
		1.2 (0.9-2.3)	n=8	**1.5** (1.0-2.2)	n=6	**1.9** (1.0-2.6)	n=7	
	AMC	0.06		0.08		0.12 (0.05-0.18)		
		0.9	n=1	2.7	n=1	3.9 (1.9-6.1)	n=10	
$CD19^+CD27^-CD38^{DIM}$	**DS**	**0.35** (0.15-0.79)		**0.17** (0.08-0.56)		**0.25** (0.08-0.37)		
		64 (41-85)	n=8	**63** (48-82)	n=6	**66** (60-71)	n=7	
	AMC	1.07		0.58		0.50 (0.18-0.56)		
		73	n=1	63	n=1	57 (47-67)	n=10	

	5 – 10 years		10 – 16 years		>16 years		DS vs AMC*	Age effect for DS + AMC**	Interaction DS and AMC†
	0.03 (0.01-0.09)		**0.01** (0.00-0.07)		**0.01** (0.01-0.02)		p=0.05	p<0.001	NS
	15 (5-28)	n=38	**14** (4-20)	n=19	**13** (10-17)	n=3	p<0.001	NS	NS
	0.04 (0.02-0.06)		0.02 (0.00-0.05)		0.03 (0.02-0.03)				
	6 (2-11)	n=8	6 (5-10)	n=11	9 (7-10)	n=2			
	0.14 (0.04-0.33)		**0.09** (0.03-0.48)		**0.08** (0.03-0.10)		p<0.001	p<0.001	p=0.006
	73 (42-86)	n=38	**77** (60-92)	n=19	**81** (69-88)	n=3	p<0.001	NS	NS
	0.42 (0.34-0.62)		0.30 (0.15-0.56)		0.23 (0.21-0.25)				
	90 (82-93)	n=8	91 (88-95)	n=11	86 (85-88)	n=2			
	0.10 (0.04-0.26)		**0.08** (0.03-0.32)		**0.06** (0.02-0.09)		p<0.001	p<0.001	p=0.017
	54 (35-87)	n=19	**75** (39-94)	n=13	**73** (63-83)	n=2	p<0.001	NS	NS
	0.38 (0.27-0.60)		0.27 (0.15-0.52)		0.20 (0.16-0.24)				
	82 (65-94)	n=8	85 (62-93)	n=11	75 (68-81)	n=2			
	0.03 (0.01-0.06)		**0.01** (0.00-0.07)		**0.01** (0.01-0.01)		p<0.001	p<0.001	p=0.041
	1.6 (0.6-3.4)	n=19	**1.0** (0.2-2.6)	n=13	**0.6** (0.4-0.8)	n=2	p<0.001	p<0.001	NS
	0.08 (0.05-0.11)		0.04 (0.01-0.15)		0.05 (0.04-0.06)				
	2.9 (1.7-4.1)	n=8	1.7 (0.8-5.0)	n=11	2.6 (2.5-2.7)	n=2			
	0.10 (0.04-0.25)		**0.07** (0.03-0.44)		**0.06** (0.02-0.09)		p<0.001	p<0.001	NS
	58 (49-80)	n=19	**67** (55-82)	n=13	**70** (57-82)	n=2	NS	p=0.001	p=0.014
	0.31 (0.24-0.42)		0.22 (0.14-0.43)		0.20 (0.18-0.22)				
	66 (59-78)	n=8	71 (59-77)	n=11	72 (68-77)	n=2			

Table 1 Continued.

		9 – 15 months		15 – 24 months		2 – 5 years		
CD19⁺CD27⁻CD38⁺	**DS**	**0.13** (0.04-0.24)		**0.06** (0.03-0.09)		**0.06** (0.02-0.08)		
		27 (7-49)	n=8	**17** (8-36)	n=6	**15** (7-21)	n=7	
	AMC	0.33		0.23		0.10 (0.05-0.31)		
		22	n=1	25	n=1	18 (7-36)	n=10	
CD19⁺CD27⁺CD38⁺⁺	**DS**	**0.02** (0.00-0.03)		**0.02** (0.00-0.03)		**0.01** (0.00-0.03)		
		3 (0-7)	n=8	**5** (2-15)	n=6	**3** (1-8)	n=7	
	AMC	0.01		0.01		0.01 (0.01-0.02)		
		0,4	n=1	1	n=1	1 (1-4)	n=10	
CD19⁺CD27⁺CD38^{DIM}	**DS**	**0.02** (0.01-0.03)		**0.01** (0.01-0.02)		**0.03** (0.01-0.04)		
		3 (2-7)	n=8	**5** (2-7)	n=6	**6** (4-10)	n=7	
	AMC	0.07		0.06		0.13 (0.01-0.20)		
		5	n=1	7	n=1	17 (1-22)	n=10	
CD19⁺κ⁺	**DS**	**0.32** (0.16-0.38)		**0.18** (0.15-0.37)		**0.23** (0.06-0.31)		
		59 (57-64)	n=6	**57** (54-60)	n=4	**60** (50-66)	n=7	
	AMC	0.85		0.49		0.44 (0.19-0.55)		
		58	n=1	56	n=1	57 (51-62)	n=9	
CD19⁺λ⁺	**DS**	**0.19** (0.11-0.25)		**0.13** (0.09-0.25)		**0.12** (0.06-0.24)		
		39 (33-42)	n=6	**40** (37-41)	n=4	**39** (36-45)	n=7	
	AMC	0.55		0.41		0.37 (0.12-0.43)		
		39	n=1	46	n=1	45 (33-52)	n=9	

	5 – 10 years		10 – 16 years		>16 years		DS vs AMC*	Age effect for DS + AMC**	Interaction DS and AMC†
	0.02 (0.01-0.10)		**0.01** (0.00-0.05)		**0.01** (0.00-0.01)		p<0.001	p<0.001	NS
	13 (5-30)	n=19	**8** (5-25)	n=13	**7** (5-9)	n=2	NS	p=0.031	NS
	0.05 (0.02-0.15)		0.04 (0.01-0.09)		0.04 (0.04-0.04)				
	12 (5-22)	n=8	12 (3-21)	n=11	14 (12-16)	n=2			
	0.01 (0.00-0.03)		**0.00** (0.00-0.01)		**0.00** (0.00-0.00)		NS	p<0.001	NS
	3 (1-17)	n=19	**2** (0-8)	n=13	**0** (0-1)	n=2	p=0.005	NS	NS
	0.01 (0.00-0.01)		0.00 (0.00-0.01)		0.01 (0.00-0.01)				
	1 (0-3)	n=8	1 (0-1)	n=11	2 (1-4)	n=2			
	0.01 (0.00-0.03)		**0.01** (0.00-0.03)		**0.01** (0.00-0.01)		p<0.001	p<0.001	p=0.018
	7 (2-11)	n=19	**6** (1-17)	n=13	**9** (5-12)	n=2	p<0.001	NS	NS
	0.07 (0.04-0.10)		0.03 (0.01-0.16)		0.02 (0.01-0.03)				
	14 (11-20)	n=8	10 (5-28)	n=11	8 (5-11)	n=2			
	0.10 (0.03-0.21)		**0.07** (0.04-0.41)		**0.05** (0.02-0.07)		p<0.001	p<0.001	NS
	62 (51-68)	n=17	**63** (58-72)	n=10	**62** (58-67)	n=2	p=0.001	NS	NS
	0.23 (0.18-0.39)		0.18 (0.10-0.35)		0.15 (0.13-0.16)				
	56 (50-64)	n=8	58 (54-65)	n=11	55 (55-55)	n=2			
	0.06 (0.02-0.14)		**0.04** (0.02-0.17)		**0.03** (0.01-0.05)		p<0.001	p<0.001	p=0.04
	36 (29-48)	n=17	**36** (31-42)	n=10	**38** (35-42)	n=2	p<0.001	NS	NS
	0.20 (0.16-0.24)		0.13 (0.08-0.25)		0.11 (0.09-0.12)				
	44 (38-50)	n=8	42 (31-45)	n=11	44 (42-46)	n=2			

Table 1 Continued.

		9 – 15 months		15 – 24 months		2 – 5 years		
κ⁺ / λ⁺ ratio	**DS**	**1.5** (1.4-2.0)	n=6	**1.4** (1.4-1.6)	n=4	**1.5** (1.1-1.8)	n=7	
	AMC	1.5	n=1	1.2	n=1	1.2 (1.0-1.9)	n=9	

Grey shaded areas: absolute numbers of B-lymphocyte subpopulations (10^9 cells/l), blank background: relative numbers (%); AMC = age-matched control children; DS = Down syndrome children; NS = not significant; * Analysis of variance, 2 to 16 years, DS versus AMC children. ** Analysis of variance, DS and AMC children together (2 to 16 years), effect of age. † Analysis of variance, interaction effect between DS and AMC children aged 2-16 years.

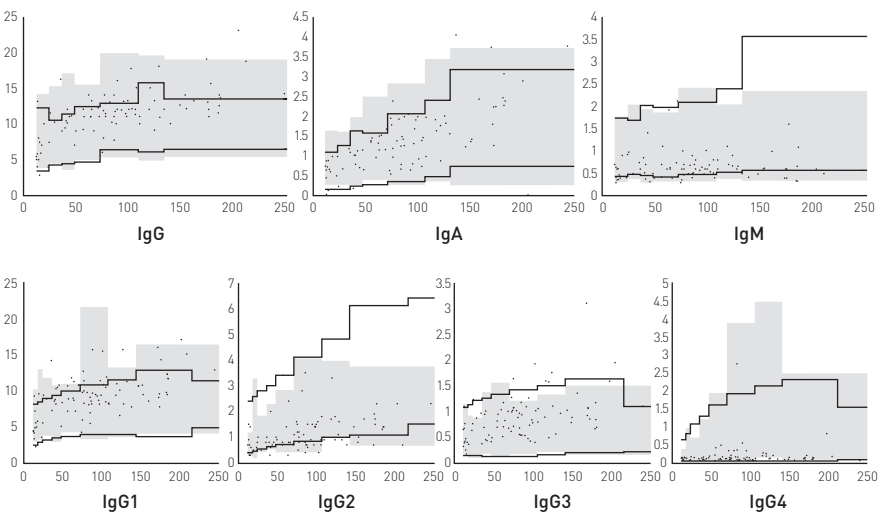

Figure 2 Immunoglobulin values in Down syndrome compared with reference values and children suffering from recurrent infections.

Values of a) IgG, b) IgA, c) IgM, d) IgG_1, e) IgG_2, f) IgG_3, and g) IgG_4 obtained in 88 children with DS are shown as *black dots*. x axis age in months; y axis immunoglobulin levels (g/L). The gray areas represent the values between the p2.5 and p97.5 of the determined immunoglobulin levels per age group in patients suffering from recurrent infections. Age-matched reference values (p2.5 and p97.5) are shown as a *black line*.

	5 – 10 years		10 – 16 years		>16 years		DS vs AMC*	Age effect for DS + AMC**	Interaction DS and AMC†
	1.7 (1.2-2.1)	n=17	**1.7** (1.4-2.3	n=10	**1.6** (1.6-1.7)	n=2	p<0.001	NS	NS
	1.3 (1.1-1.6)	n=8	1.4 (1.2-2.1)	n=11	1.3 (1.2-1.3)	n=2			

Table 2 Clinical features.

	9 – 15 mo (n=11)	15 – 24 mo (n=4)	2 – 5 y (n=16)	5 – 10 y (n=38)	10 – 16 y (n=19)	>16 y (n=3)	Total (n=91)
No increased infection rate (all ages)	n=5		n=6	n=5	n=1	n=2	n=19
Increased - mainly respiratory - infection rate (age at inclusion <8 years)	n=6	n=4	n=10	n=20			n=40
Increased - mainly respiratory - infection rate until, but not after, the age of 8 years				n=6	n=12		n=18
Increased - mainly respiratory - infection rate (age at inclusion >8 years)				n=7	n=6	n=1	n=14
Celiac disease	0/11**	1/4	1/16	2/38	1/19	0/3	5/91
Auto-immune hypothyroidism	0/11	1/4	0/16	1/38	1/19	0/3	3/91

	9 – 15 mo (n=8)	15 – 24 mo (n=6)	2 – 5 y (n=7)	5 – 10 y (n=19)	10 – 16 y (n=13)	>16 y (n=2)	Total (n=55)
Asthma	4/8*	3/6*	0/7	3/19	0/13	0/2	10/55
Allergy	1/8	1/6	0/7	0/19	0/13	0/2	2/55
Total IgE elevated	0/6**	1/3	1/5	2/17	2/12	0/1	6/44
Specific IgE inhaled allergens present	0/2	0/0	0/3	0/12	0/7	0/1	0/25
Specific IgE food allergens present	0/3	0/2	0/4	0/12	0/6	0/1	0/28

*It is doubtful whether these children will continue to have the diagnosis of asthma in later years [21]. **0/11 means 0 of 11 patients tested, etc.

DISCUSSION

The profound B-lymphocytopenia in DS children, with decreased transitional and naive B-lymphocytes compared with AMC children, is the most striking result of our study. There are no indications for release of unusual numbers of "immature" B-lymphocytes from the bone marrow ($CD10^+$, $CD20^-$), the cells show the normal phenotype of the transitional and naive stages of peripheral B-lymphocyte development [22]. As stated before, this could be caused by decreased B-lymphocyte proliferation due to a disturbance in T-lymphocyte help, an intrinsic B-lymphocyte defect, or a combination of these.

Interestingly, the distribution of B-lymphocyte subpopulations is reminiscent of the situation found in patients with common variable immunodeficiency (CVID) [23-25]: $CD27^+$ cells, $CD21^{high}$ cells and $CD23^+$ cells are decreased in absolute and relative numbers in the children with DS. These findings are suggestive of an intrinsic defect in B-lymphocyte maturation in the periphery.

CD21 is the complement type 2 receptor; it has a role in the response to polysaccharide antigens like pneumococcal capsular elements. These antigens form a complex with CD21 on B-lymphocytes causing a T-cell-independent response. The lower response to unconjugated pneumococcal vaccination and the increased rate of respiratory infections in DS could be related to this decreased expression of CD21. Interestingly, a subgroup of patients with CVID with relatively increased $CD21^{low}$ B-lymphocytes is more likely to develop splenomegaly, auto-immune diseases and lower respiratory tract infections [26]; the latter two are frequently found in DS as well.

CD23 is the low-affinity IgE-receptor (FcεRII), it is a ligand of CD21. Together, they stimulate B-lymphocyte proliferation and differentiation [27]. CD23-expression is increased just before the class-switch from IgM to IgG, IgA or IgE [27]. Besides, CD23 is involved in both positive and negative feedback-loops for IgE-homeostasis [27]. Interestingly, both asthma incidence (RR 0.4, 95% CI 0.2-0.6) and IgE-levels are decreased in DS [28, 29], which is consistent with our findings. Our results suggest that increased IgE production is associated with a higher level of CD23 expression in children with DS.

The serum Ig values in children with DS – with or without recurrent infections – and non-DS children with recurrent infections are both abnormal, but differ from each other. Decreased IgG_2 is a well-known abnormality in children with recurrent infections; this coincides with our findings in children with DS. However, the increased IgG, IgG_1, and IgG_3, and decreased IgM and IgG_4 are found in the children with DS only.

In conclusion, we found that the humoral immune system is disturbed in children with DS. We could not differentiate between an intrinsic B-lymphocyte defect and disturbed T-lymphocyte help as the most important cause based on our present data. This question remains unanswered, and further studies are needed to solve it.

Acknowledgments

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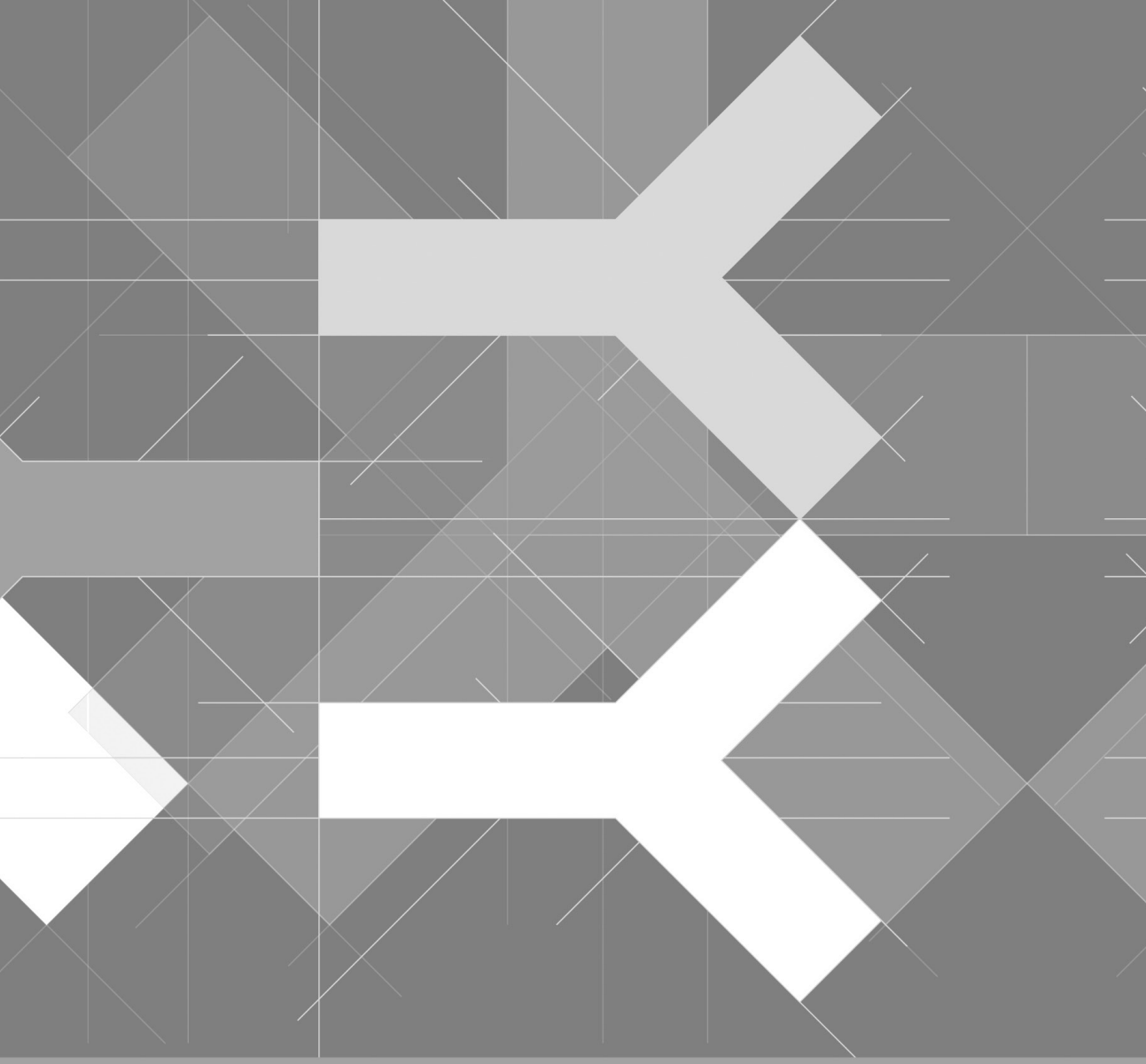

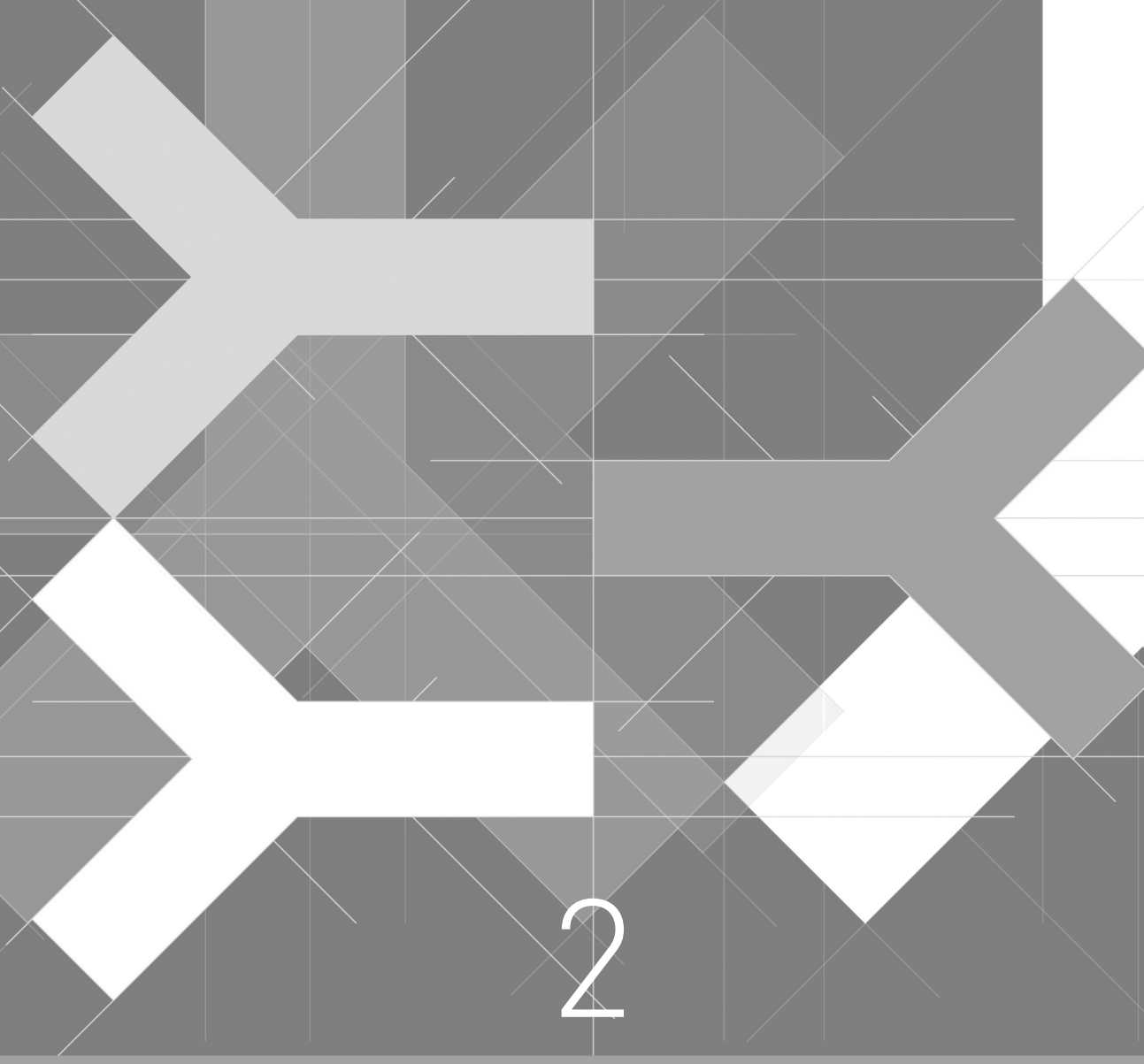

2

VACCINATION RESPONSE STUDIES

6

IMPAIRED AVIDITY MATURATION AFTER TETANUS TOXOID BOOSTER IN CHILDREN WITH DOWN SYNDROME

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ABSTRACT

Down syndrome children show a decreased avidity of the antibody response after tetanus toxoid booster vaccination at nine years of age suggesting impaired memory B cell selection in the germinal center. Clinicians need to be aware of this ongoing subtle immunological deficit in Down syndrome.

INTRODUCTION

Children with Down syndrome show abnormal thymocyte maturation and T-lymphocyte development [1]. The increased frequency of haematological malignancies and auto-immune diseases but absence of opportunistic infections in DS suggests this is associated with a clinically relevant but mild T-lymphocyte deficiency [2]. The increased, mainly respiratory, infections in especially younger children with DS suggest a possible B-lymphocyte problem [3]. Recently, we found a striking B-lymphocytopenia in DS [4], and decreased immunoglobulin(Ig)G_2, IgG_4 and IgM but increased IgG_1, IgG_3 and total IgG serum levels after 5 years of age [3], as had been described before [5]. It is not clear whether the low absolute B-lymphocyte numbers and their altered Ig-production are mainly caused by an intrinsic B-lymphocyte defect, which would fit the increased frequency of respiratory infections in DS [5], or whether these changes are mainly accounted for by a T-lymphocyte defect leading to altered T-lymphocyte help and impaired T-B-lymphocyte collaboration.

Assaying specific antibody production against well-defined antigens can be used as an in vivo model to assess the capacity of the immune system. The response to T-cell dependent antigens represents the combined outcome parameter of T-lymphocyte function, T-B interaction and B-lymphocyte function, whereas the response to T-cell independent antigens is largely if not only determined by B-lymphocyte function alone. Repeated vaccination with a T-cell dependent antigen activates selected clones of memory B-cells that produce high avidity immunoglobulins after somatic hypermutation.

A strong T-cell-dependent B-lymphocyte response is normally seen after exposure to tetanus toxoid (TT), a highly immunogenic protein antigen, with increased avidity after repeated boosting [6]. Disturbed responses may be indicative of a clinically relevant antibody deficiency. Earlier reports on tetanus vaccination in DS showed the ability to achieve the threshold of protective antibody levels at a random time point after vaccination, which is set at 0.2 IU/ml (= 1 µg/ml) specific anti-TT-IgG according to World Health Organization criteria [7,8]. However, peak anti-TT-responses at 3 to 4 weeks after booster vaccination and avidity maturation data, which will be disturbed in case of impaired T-B interaction processes in the germinal center, have never been reported in DS.

PATIENTS AND METHODS

TT is part of the Dutch immunization program at 2, 3, 4 and 11 months of age, with boosters at 4 and 9 years. In a cross-sectional study, paired sera of 22 children with DS (4 years of age, n=15; 9 years of age, n=7) were obtained before and 3 to 4 weeks after

regular TT-booster at 4 or 9 years of age (1 pre-vaccination serum missing at 4 years of age). Specific anti-TT-IgG and IgG-subclasses were measured by an antibody-capture enzyme-linked immunosorbent assay [9]. Since the IgG-response after TT-booster in healthy controls predominantly (>75%) consists of IgG_1 antibodies, the anti-TT avidity-ELISA was performed for IgG_1 only [9]. Avidity is expressed as the relative index representing the molarity of NaSCN required to elute 50% of TT-bound IgG_1 [9]. Results were compared with reference values obtained in 20 healthy age-matched controls (for the children with DS at 4 years of age) and 20 healthy adults (Ad-REF) (for the children with DS at 9 years of age), respectively [6]. Statistical analysis was performed after logarithmic transformation using the *t* test and SPSS 16.0 for Windows.

RESULTS

In our DS cohort, 10 of 14 (71%) of the children at 4 years and all 7 children (100%) at 9 years of age already had protective antibody titers according to the WHO-criteria before their TT-booster vaccination. All 22 children with DS (100%) responded to the booster vaccination with an increase in antibody titer. Post-vaccination antibody formation in DS and controls consisted predominantly of IgG_1 (about 75%), as expected. However, post-booster vaccination anti-TT-antibody titers (Figure 1a) and IgG_1-avidity (Figure 1c) were significantly lower in 4-year-old children with DS compared with AMC children: anti-TT-total IgG geometric mean titer (GMT) was 58 µg/ml (range 32-164) in DS and 178 µg/ml (range, 72-424) in AMC ($p<0.001$), anti-TT-IgG_1 GMT was 45 µg/ml (range 17-130) in DS and 131 µg/ml (range, 26-380) in AMC ($p<0.001$), anti-TT-IgG_2 GMT was 0.60 µg/ml (range, 0.14-10) in DS and 4.0 µg/ml (range, 0.62-30) in AMC ($p<0.001$), anti-TT-IgG_3 GMT was 2.4 µg/ml (range, 0.49-19) in DS and 1.5 µg/ml (range, 0.20-17) in AMC ($p=0.248$) and anti-TT-IgG_4 GMT was 0.26 µg/ml (range, 0.02-22) in DS and 2.0 µg/ml (range, 0.08-21) in AMC ($p=0.005$) (Figure 1a). IgG_1-avidity GMT was 2.5 (range, 2.2-2.9) in DS and 2.8 (range, 2.6-3.1) in AMC children ($p=0.002$). The fold increase of post- versus pre-booster anti-TT-IgG1 was not significantly different between DS and AMC children at 4 years of age.

After booster vaccination at 9 years of age, anti-TT-antibody total IgG, IgG_1, and IgG_3 GMT (Figure 1b) fall within the adult reference range, but anti-TT-IgG_2 GMT and anti-TT-IgG_4 GMT remained significantly decreased: anti-TT-IgG_2 GMT was 0.47 µg/ml (range, 0.14-4.5) in DS and 2.3 µg/ml (range, 0.47-24) in Ad-REF ($p=0.011$), anti-TT-IgG_4 GMT was 0.14 µg/ml (range, 0.03-3.5) in DS and 1.4 µg/ml (range, 0.09-28) in Ad-REF ($p=0.015$). IgG_1-avidity (Figure 1c) is still significantly decreased: GMT was 2.7 in DS and 2.9 (range, 2.7-3.2) in Ad-REF ($p=0.006$). The fold increase of post- versus prebooster anti-TT-antibody titers was not significantly different between DS children at 9 years of age and Ad-REF.

Conform previous observations in healthy controls [6], there was no correlation between the titer and the avidity of anti-TT-IgG1 postvaccination in the children with DS.

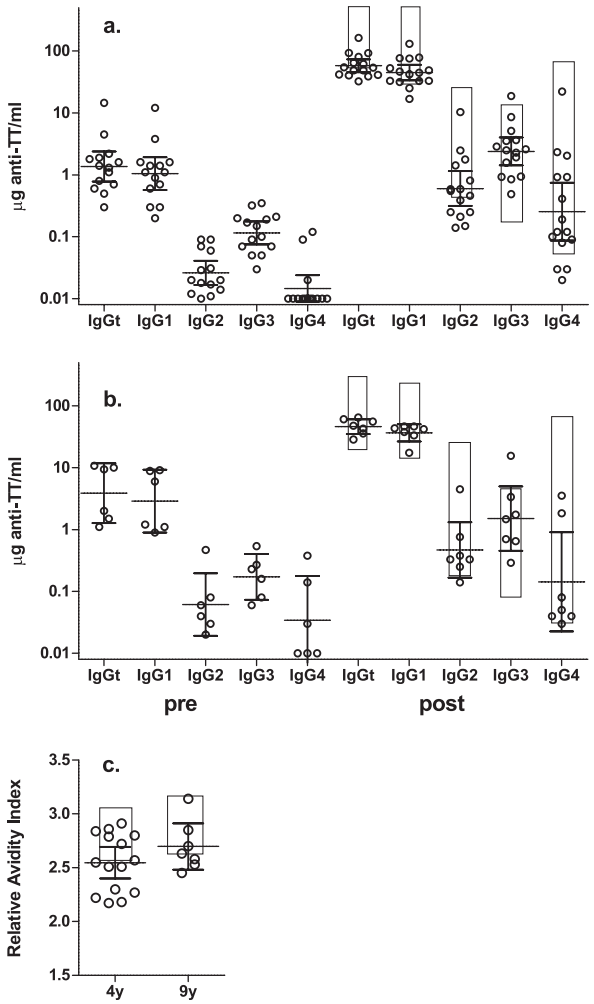

Figure 1 Specific anti-TT IgG and IgG-subclasses before and 3-4 weeks after tetanus booster vaccination at 4 years (A) and 9 years (B) of age; anti-TT IgG1 avidity after tetanus booster vaccination at 4 or 9 years of age (C). anti-TT indicates antitetanus toxoid.

IgGt, total anti-TT IgG. Relative avidity index: the molarity of NaSCN required to elute 50% of TT-bound IgG_1. Open circles: Down syndrome subjects. Horizontal lines: geometric mean Down syndrome children (95% CI); Open bars:reference group (+/- 2SD).

DISCUSSION

We found a decreased anti-TT humoral immune response in a cohort of 22 children with DS: IgG and IgG-subclass titers are lower at 4 years of age, and – despite normalisation of IgG, IgG_1, and IgG_3 titers - avidity maturation remains impaired at 9 years of age. These data show that DS children have lasting difficulties with specific anti-TT-antibody formation, although 'protective levels' for tetanus toxoid are being reached.

We compared our data to other possibly immunocompromised patient cohorts from the literature. Patients with decreased numbers of helper-T-lymphocytes such as children with partial DiGeorge syndrome [10] and HIV^+ children [11] and adults [9] mount a protective response but with lower mean IgG anti-TT-antibody titers. There are no pediatric data on antibody avidity, but IgG_1-avidity in HIV^+ adults was normal after TT-booster vaccination [9,11]. The decreased TT-specific IgG_1-avidity levels despite repeated booster vaccination are suggestive of a subtle impairment of the selection process of memory B-cells in the germinal center in DS, which cannot be overcome by repeated TT- booster vaccination and maturation of the DS immune system with ageing. This is reminiscent of patients with common variable immunodeficiency in whom insufficient somatic hypermutation leading to reduced generation of high-affinitiy antibodies has been described, associated with an increased frequency of severe respiratory tract infections [12,13].

Clinicians need to be aware of this ongoing deficiency in antiprotein antibody quality in DS.

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7

INFLUENZA A/H1N1 VACCINATION RESPONSE IS INADEQUATE IN DOWN SYNDROME CHILDREN WHEN THE LATEST CUT-OFF VALUES ARE USED

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ABSTRACT

We determined the response of 48 Down syndrome children to 2 doses of influenza A/H1N1 vaccination. 92% reached the previously defined protective level (HI titer ≥1:40), but only 27% reached the level of ≥1:110 which was recently described to predict the conventional 50% clinical protection rate in children. Further studies, and potentially adaptations of the schedule, are needed.

INTRODUCTION

Many children with Down syndrome (DS) suffer from recurrent respiratory infections with higher rates of hospitalisation, sepsis and death [1,2]. This can be explained by anatomic and functional ear-nose-throat abnormalities, hypotonia, cardiac abnormalities, mental retardation and increased incidence of gastroesophageal reflux [3,4,5], but also decreased antibody responses to vaccination are seen [6,7,8]. The immune response to influenza vaccination in DS has been described as decreased [9] or as normal [10].

In 2009, the emergence of a new influenza A virus (H1N1) led to a worldwide pandemic. Vaccination campaigns were started in response to the expected high morbidity and mortality. In the Netherlands, patients with e.g. chronic respiratory, cardiac or immunological diseases who normally would be offered an annual seasonal influenza vaccine were offered H1N1 vaccination as well. Also, healthy children aged 6 months to 4 years were offered two influenza A/H1N1 vaccinations in a nation-wide campaign. DS children are not regarded as a risk group for influenza in the Netherlands, and therefore are not routinely offered a seasonal influenza vaccine, unless they have additional pulmonary or cardiac disease. The 2009 H1N1 vaccination campaign was used to measure the antibody response following 2 doses of influenza A/H1N1 in 48 DS children.

METHODS

Three Dutch hospitals - Jeroen Bosch Hospital in 's-Hertogenbosch, Maxima Medical Centre in Veldhoven, Elkerliek Hospital in Helmond - included 73 DS children after informed parental consent. During routine visits to the outpatient clinic extra blood was drawn. Forty-eight blood samples were collected from 48 vaccinated DS children (median age 8.8, range 0.7-17 years; 26 boys) with a median of 154 days (range 23-267) after two doses of the 2009 influenza A/H1N1 vaccine (monovalent MF59-adjuvanted). A comparison was made with 25 unvaccinated DS children (median age 8.5, range 0.2-19 years; 17 boys). All assays were performed in the laboratory of the St. Elisabeth Hospital in Tilburg, the Netherlands. Virus-specific antibodies were measured by a hemagglutination-inhibition (HI) assay, using egg-grown A/California/7/2009 A (H1N1) pandemic virus and fresh turkey red blood cells in Alsever's solution (Biotrading, Netherlands), according to standard methods [11]. The HI titer was the reciprocal of the highest dilution of serum that inhibited virus-induced hemagluttination. Titers <10 were assigned a value of 5. Comparison of HI assay data from different laboratories is complicated by a lack of standardization due to the use of various influenza virus strains, different receptor-destroying enzymes (homemade or commercially bought)

and also by differences in quality and nature of red blood cells. Red blood cells and receptor-destroying enzyme were bought commercially to overcome this problem and a candidate international standard for antibody titers to pandemic H1N1 virus was used to calibrate our HI titers [12].

A HI titer of ≥1:40 has been reported to result in a 50% reduction of influenza infections in healthy adult individuals [13]. Up to recently, this titer was used as a protective cut-off value in children as well. Recently, Black et al [14] challenged this cut-off value in healthy children and suggested a new cut-off value of at least ≥1:110 in healthy children for a 50% influenza infection reduction.

RESULTS

HI titers of DS children are shown in figure 1**.** Ninety-two percent of the 48 vaccinated DS children reached a HI titer of ≥1:40. Only 27% of the 48 vaccinated DS children reached a HI titer of ≥1:110. In comparison, 28% of unvaccinated DS children reached a HI titer of ≥1:40 after the influenza season, and no unvaccinated DS child reached the HI titer of ≥1:110.

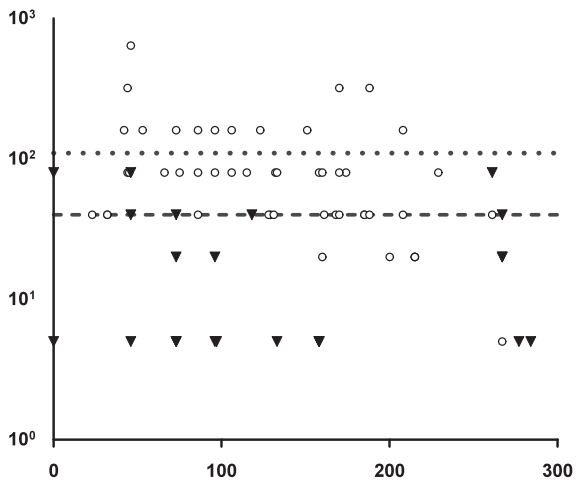

Figure 1 Hemagglutination-inhibition titer in children with Down syndrome after influenza A/H1N1 vaccination.

X-axis: days after second H1N1 vaccination. Y-axis: HI titer (1:y). Dots: Down Syndrome (DS) children vaccinated twice with influenza A/H1N1. Triangles: unvaccinated DS children. Horizontal dotted line: HI titer = 1:110. Horizontal striped line: HI titer = 1:40.

In one child, parents reported a possible side-effect of fever after the first vaccination. In the vaccinated group flu-like symptoms were reported in one child, but no influenza tests were performed during that time. In the unvaccinated group four children had flu-like symptoms; one child was admitted to hospital (test-results: Influenza negative, Respiratory Syncytial virus positive).

DISCUSSION

Post-vaccination hemagglutination-inhibition titers of ≥1:40 have been reported to result in a 50% reduction of influenza infections in healthy adults [13]. Since 92% of our 48 vaccinated DS children reached this HI titer after two doses of the 2009 influenza A/H1N1 vaccine, it would be logical to conclude that this is an effective vaccination strategy for DS children. However, this HI cut-off value has not been studied for influenza A/H1N1 vaccination in children. Pre-vaccination data from the available literature show that up to 30% of healthy children already reach a HI titer of ≥1:40 without a history of previous influenza A/H1N1 vaccination or active influenza A/H1N1 infection [15,16]. Cross-reactive antibodies from other influenza-strains could be held accountable, but it seems that this explanation is not entirely valid, as recent studies demonstrated no cross-reactivity between antibodies of previous seasonal influenza- and H1N1-strains in children [17,18].

The applicability in children of the WHO cut-off value has recently been challenged by Black et al. in this journal [14]. They predict a HI titer of ≥1:110 is needed for a 50% clinical protection rate in healthy children, at least until 6 years of age. Unfortunately, most pediatric publications do not show individual HI titers. In a recent Swiss publication [19], post-vaccination HI titers of ≥1:110 were reached in ≥90% of healthy children using one dose of influenza A/H1N1 MF59-adjuvanted vaccine. No large studies correlating HI titer and clinical protection are as yet available in children.

Our study shows that DS children only reach the new proposed cut-off value of ≥1:110 in 27% of the cases studied. This is a lower response than was reported for non-DS children, and is another example of decreased immunological vaccination response in DS. A larger study is needed to determine the efficacy of clinical protection of H1N1 influenza vaccination in DS children.

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8

DECREASED RESPONSE AFTER CONJUGATED MENINGOCOCCAL SEROGROUP C VACCINATION IN CHILDREN WITH DOWN SYNDROME

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INTRODUCTION

Meningococcal serogroup C conjugate (MenC) vaccine is part of the Dutch immunization program since 2002. In the MenC vaccine, the polysaccharide antigen is linked to the protein carrier tetanus toxoid with the aim to achieve an adequate immune response at an early age, which would not be possible with a pure polysaccharide vaccine. The immune response to a conjugate vaccine is characterized by T-cell-dependent isotype-switching to IgG-antibodies, especially IgG_1, and induction of immunological memory.

METHODS

In a catch-up campaign, all children (1-18 years) were offered a single dose of MenC in the Netherlands in 2002. Blood samples of 19 Down syndrome (DS) children (mean age 10.6, range 5.3-17.4 years) were taken during regular hospital visits 3 months (n=7; mean 13 weeks, range 39-107 days), or around 1 year (n=12; mean 50 weeks, range 275-447 days), after this single dose of MenC vaccination. MenC polysaccharide (PS) specific IgG, IgM and IgA levels were measured using an antibody-capture enzyme-linked immunosorbent assay.[1] Results were compared with reference values of healthy adults from the same laboratory, 1 month (n=12) and 1 year (n=11) after single MenC vaccination.

RESULTS

At 3 months post-vaccination, geometric mean MenC/PS specific IgG, IgA and IgM serum levels were 5.5 (range 1.4-41), 0.71 (0.03-11) and 0.61 (0.10-7.5) µg/mL, compared to 26 (5.6-59), 5.6 (2.1-11) and 5.2 (1.7-35) µg/mL in healthy controls (p= 0.014, 0.028, 0.12 assessed by Mann-Whitney test respectively). One year after vaccination, geometric mean MenC/PS specific IgG, IgA, and IgM levels were 2.7 (0.78-15), 0.19 (0.02-1.2) and 0.28 (0.15-0.76) µg/mL, whereas reference values were 4.5 (0.68-19), 0.79 (0.13-3.8), and 0.71 (0.16-5.1) µg/mL (p= 0.204, 0.019, <0.001 assessed by Mann-Whitney test respectively). The 19 DS children did reach protective, but lower levels after a single MenC vaccination in comparison to healthy adults, despite the fact that 9 of them showed hypergammaglobulinemia according to age-matched reference values.[2]

CONCLUSIONS

Assaying specific antibody production against well-defined antigens can be used as a model to assess T-cell-dependent (anti-protein) and T-cell-independent (anti-polysaccharide) antibody responses; conjugated protein-polysaccharide vaccines like MenC show aspects of both types of responses. Impaired specific antibody responses to unconjugated pneumococcal polysaccharide have been described in DS, suggesting a B-lymphocyte problem.[3] Impaired responses to influenza, hepatitis B and tetanus (protein-prototype) have been described as well, suggesting an additional T-lymphocyte or T-B-interaction problem.[3] Not unexpectedly therefore, our data show that protein conjugation does not fully overcome the impaired antibody production to this polysaccharide antigen in DS.

Of course, decreased antibody production upon vaccination may have clinical implications as well. DS children have frequent respiratory infections, but an increased frequency of meningococcal disease has not been described in recent surveys, either in the pre-[4] or post-MenC[5] era, but specific attention was not given to this subject. So, it is as yet unclear whether children with Down syndrome are at greater risk of meningococcal disease, or whether they would benefit from an additional dose of conjugated meningococcal vaccination. Further studies are needed to elucidate this.

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9

FUNCTIONALITY OF THE PNEUMOCOCCAL ANTIBODY RESPONSE IN DOWN SYNDROME SUBJECTS

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ABSTRACT

We investigated the anti-polysaccharide antibody responses in subjects with Down syndrome (DS) because DS subjects show decreased peripheral B-lymphocyte numbers in all age groups, and a clinical picture of recurrent respiratory tract infections and increased incidence of autoimmune diseases which is reminiscent of common variable immunodeficiency disorders (CVID)-like disease. We determined titers and opsonophagocytosis in response to conjugated and unconjugated pneumococcal serotypes in 18 DS subjects aged 6 to 24 years. The results show adequate serotype-specific antibody titers in response to all conjugated and almost all unconjugated serotypes used. Opsonophagocytosis activity as measured against pneumococcal serotypes 9N, 19F and 23F was also found to be intact. We conclude that DS subjects do not have a clear defect in their anti-polysaccharide antibody response.

INTRODUCTION

Down syndrome, or trisomy 21, is a relatively common chromosomal disorder (incidence 1:800) [1] associated with a variety of immunological abnormalities including abnormal thymocyte maturation and T-lymphocyte development [2], and a striking B-lymphocytopenia [3] with decreased immunoglobulin(Ig)G_2 and IgG_4 and increased total IgG-serum levels after 5 years of age [4]. Lower specific antibody responses have been reported: the antibody response [5] and avidity maturation after tetanus toxoid booster [6] is impaired, the antibody responses to hepatitis B vaccination [7] and the hemagglutination-inhibition titer to influenza A/H1N1 vaccination [7,8] are decreased.

DS subjects have an increased incidence of mainly respiratory infections [9,10], which are often caused by *Streptococcus pneumoniae*, a polysaccharide-encapsulated bacterium. This might be related to a (subtle) antibody deficiency. However, the frequent infections may also be – at least partially – explained by the facial anatomy, chronic Eustachian tube dysfunction, and hypotonia in DS as well [10,11].

An adequate immune response to *S. pneumoniae* depends on the production of serotype-specific antibodies, but in particular on the functionality of these antibodies (opsonophagocytosis). Younger children are not yet able to produce anti-polysaccharide antibodies; protein-conjugated vaccines are used to overcome the effects of this physiological immaturity of the anti-polysaccharide antibody response in infants. Specific anti-polysaccharide antibody deficiency (SPAD) is a well-known milder antibody deficiency in non-DS older children and adults [14], which may lead to an increased frequency of respiratory infections.

The antibody response to the unconjugated 23-valent polysaccharide pneumococcal vaccine was reported lower but not deficient in DS by Costa-Carvalho et al. [12] and Nurmi et al. [13] after a single vaccination as compared to healthy controls in children >6 years [12] and adults [13], respectively. It has not yet been established whether DS subjects react normally to protein-conjugated pneumococcal vaccinations, which are now part of the regular immunisation scheme in many countries. Adequate pneumococcal vaccination could be clinically relevant because of the tendency in DS towards recurrent respiratory tract infections. Until now, DS infants are offered the same immunisation schedule for pneumococci as healthy infants, a scheme generally containing four doses (3+1) of a conjugated pneumococcal vaccine. In the Netherlands, the 7-valent conjugated pneumococcal vaccine Prevnar® (PCV-7) was introduced in the national immunisation program for infants born after April 2006. As of March 2011, it has been replaced by the 10-valent conjugated pneumococcal vaccine Synflorix®.

Given the immunological disturbances and clinical phenotype in DS, it is attractive to hypothesize that SPAD could – at least in part – explain the recurrent respiratory

infections in DS. We investigated this using polysaccharide and conjugated pneumococcal vaccines, which are prototypes for T-cell independent (TI) anti-polysaccharide and T-cell dependent (TD) combined anti-protein and anti-polysaccharide antibody responses, respectively.

We used a vaccination scheme with conjugated followed by polysaccharide pneumococcal vaccine in 18 DS subjects. The response to polysaccharide vaccine only serotypes were used to analyze whether SPAD is an integral feature of the DS immune system. The combined polysaccharide / conjugated vaccine serotypes were used to analyze whether SPAD, if found, can be overcome by protein-conjugated vaccination. The study was restricted to participants >5 years of age, because young children would be expected to show a physiological 'SPAD' anyway.

Results from non-DS healthy adults and non-DS children with recurrent – mainly respiratory – infections without serious primary immunodeficiency were used to compare the serotype-specific pneumococcal antibody response profiles.

MATERIAL AND METHODS

This study was approved by the local Medical Ethical Committee, and was performed in 2009 and 2010 in the Jeroen Bosch Hospital, 's-Hertogenbosch, the Netherlands. These subjects were born before 2006, before the introduction of pneumococcal vaccination in the national Dutch immunisation program. Eighteen non-institutionalized Dutch DS subjects >5 years of age who had never received a pneumococcal vaccine were included after parental informed consent (median age at inclusion 12 years, range 6-24 years; 10 female, 3 subjects aged >18 years). Data on the history of infections, and incidence of autoimmune diseases and malignancies were collected from the medical files and verified with the parents.

DS subjects were vaccinated twice with a heptavalent pneumococcal conjugate vaccine (PCV-7; Prevnar®, Wyeth, Rochester, NY, USA) at t=0 and t=4 weeks, followed by a single vaccination with a 23-valent pneumococcal polysaccharide vaccine (PPV-23; Pneumo23®, Pasteur-Merieux, Lyon, France) at t=12 weeks. PCV-7 contains 2µg of serotype 4, 9V, 14, 19F and 23F polysaccharide, 4µg of serotype 6B polysaccharide, and 2µg of serotype 18C oligosaccharide, each conjugated individually to the CRM_{197} protein. PPV-23 contains 25µg of serotype 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, and 33F polysaccharide. Blood samples were taken before the first and four weeks after the last vaccination. Serum samples were stored at –20 °C until further analysis.

Anti-pneumococcal IgG-antibodies against pneumococcal serotypes 1, 3, 7F, 8, 9N, 12F, and 19A (PPV-23 only) and serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F (both PPV-23 and PCV-7) were analysed by a multiplex bead-based assay (Luminex Cooperation, Austin,

TX, USA) according to the manufacturer's instructions [18]. Data are presented as individual IgG-antibody titers (µg/ml) to each of the fourteen pneumococcal serotypes tested.

For the prevention of invasive pneumococcal disease (IPD) in children a cut-off value of ≥0.35µg/ml was agreed upon by the WHO [15]. For the prevention of mucosal infection in children after pneumococcal vaccines a higher cut-off value of ≥1.0µg/ml has been suggested [16,17]. In the data analysis we used this higher cut-off value because an important goal in DS children would also be the prevention of their recurrent respiratory tract infections.

Antibody titers to serotype 3 (PPV-23-only) were compared to single-PPV23 vaccinated healthy adults (n=15, median age 42 years, range 22-69 years) and children with recurrent respiratory infections but without serious primary immunodeficiency (n=17; median age 6 years, range 2-18 years). Antibody titers to serotype 1, 7F, 8, 9N, 12F, and 19A (PPV-23-only) were compared to single-PPV-23 vaccinated children with recurrent respiratory infections (n=14; 3 missing due to insufficient amount of serum) from the same laboratory and to single PPV-23 vaccinated healthy Belgian adults (n=40; mean age 21 years, range 19-30 years) [19] and children (n=35, mean age 8 years, range 3-15 years) [19] and Belgian elderly (n=58, mean age 76 years, range 58-92 years) [20] from the literature using the same multiplex bead-based assay.

Antibody titers to serotype 4, 6B, 9V, 14, 18C, 19F, and 23F (both PCV-7 and PPV-23 serotypes) were compared to English children with recurrent infections but without serious primary immunodeficiency who underwent the same combined vaccination schedule using the same multiplex bead-based assay (n=25; median age 5 years, range 2-16 years; original data kindly provided by R. Borrow) [21].

Opsonophagocytosis assays (OPAs) for serotype 9N (PPV-23-only), 19F and 23F (both PPV-23 and PCV-7) were performed in DS subjects and single-PPV-23 vaccinated children with recurrent respiratory infections from the same laboratory. These three serotypes were chosen because the DS subjects responded with low (9N, unconjugated), medium (19F, conjugated) and high (23F, conjugated vaccine) antibody titers to these serotypes. A minimum antibody titer of 0.35µg/ml is needed for this OPA. In brief, serum samples (100µl) were diluted in antibody depleted human serum (ADHS) to a final concentration of 0.35µg/ml. *S. pneumoniae* strains of serotype 9N, 19F and 23F were cultured, labeled with fluorescein isothiocyanate (FITC), and adjusted to a concentration of 2.5 x 10^8/ml. Equal volumes of FITC-labeled pneumococci were added to the sera. Peripheral blood granulocytes, obtained from healthy adult volunteers, were prepared by osmotic lysis of whole blood. A suspension of 5 x 10^6 granulocytes per ml was prepared in Roswell Park Memorial Institute medium (RPMI 1640 + GlutaMax T -I, 1x 0.1% Clindamycin) + 10% fetal calf serum. Opsonized pneumococci and 100µl granulocytes were incubated for 15 minutes at 37˚C; incubation was stopped by addition of ice-cold phosphate-buffered saline. Cells were

washed twice with cold RPMI and measured on a fluorescence-activated cell sorting (FACS) Calibur flow cytometer (Becton Dickinson, San Jose, CA). From each sample, 10,000 cells were measured and the FITC fluorescence intensity of the gated granulocytes was used as parameter for phagocytosis. Data are expressed as phagocytosis index. The phagocytosis index (PI) is the percentage of granulocytes that have phagocytized multiplied by the mean fluorescence intensity (MFI).

All concentrations of anti-pneumococcal IgG-antibodies pre- and post-vaccination and OPA titers were transformed by the logarithmic function. Differences in antibody response and OPA titers between groups were evaluated by the Welch *t* test. A p-value of <0.05 was considered statistically significant.

RESULTS

The clinical characteristics of the DS subjects are listed in Table 1. DS anti-pneumococcal IgG-antibodies pre- and post-vaccination are shown in Table 2. Post-vaccination IgG-antibodies of all non-DS groups (see Methods) are shown in Table 2 as well. Table 3 shows the percentage with a seroprotective response (cut-off value ≥1.0µg/ml) for each serotype in DS subjects and non-DS subjects (healthy adults, and children with recurrent infections from the same laboratory, and children with recurrent infections [21], original data kindly provided by R. Borrow). Post-vaccination anti-pneumococcal IgG-antibodies of DS subjects and English children with recurrent infections [21] undergoing the same vaccination scheme are shown in Figure 1. The OPA results for the DS subjects and the non-DS subjects with recurrent respiratory infections (but without serious primary immunodeficiency) are shown in Figure 2.

Table 1 Clinical features of Down syndrome subjects.

	Total (n=18)
No recurrent infections	4 (24%)
History of recurrent respiratory infections	14 (78%)
Recurrent respiratory infections > 6 years	8 (44%)
Celiac disease	3 (17%)
Auto-immune hypothyroidism	4 (22%)
Asthma after age of 6 years	5 (31%)

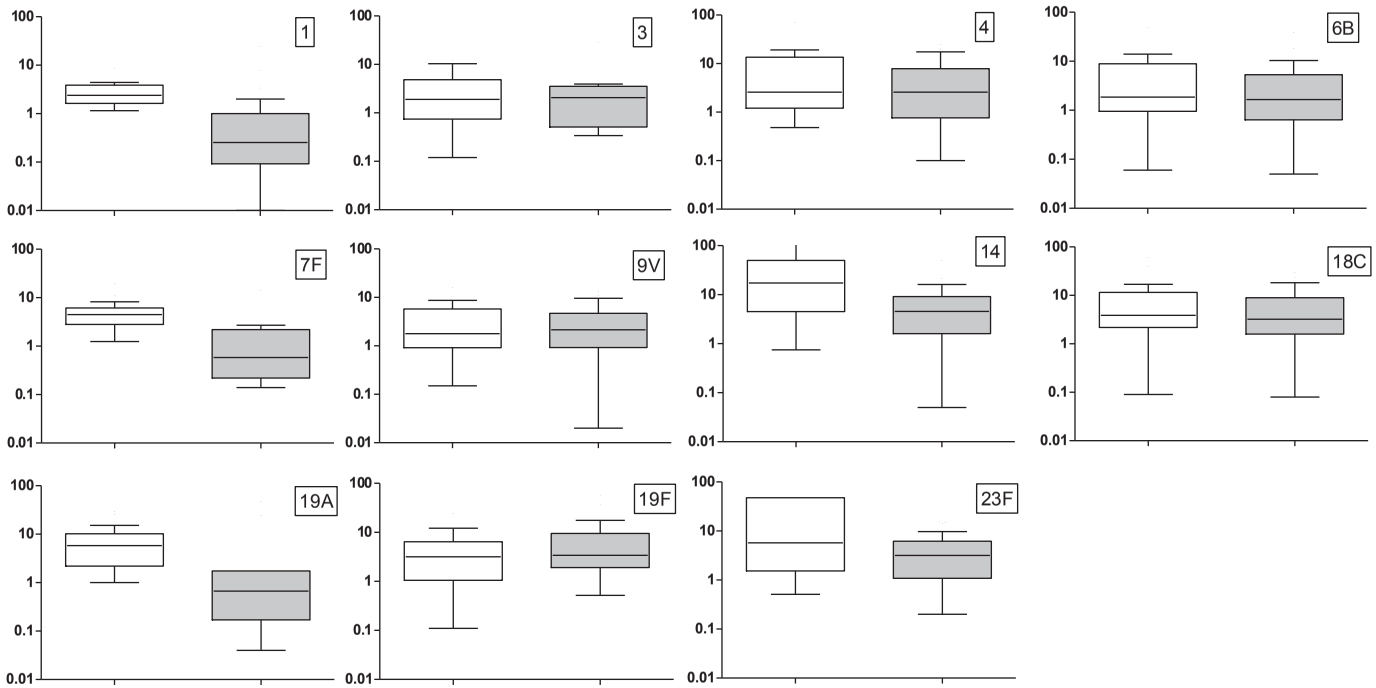

Figure 1 Response to serotypes 1, 3, 4, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F.

Geometric mean antibody titer (µg/ml), logarithmic scale; 25th and 75th percentiles. Subjects with DS (white bars) compared to children without DS with the same vaccination scheme (grey bars) [21].

Table 2 Anti-pneumococcal IgG titers in subjects with DS compared to healthy children and adults and children with recurrent infections.

	Down syndrome subjects (N=18)		Healthy adults (N=15)	
Vaccination scheme	**2x PCV-7 and 1x PPV-23**		**1x PPV-23**	
Serotypes	**Pre-GMT**	**Post-GMT**	**Post-GMT**	
PS3	0.27 (0.09-0.60)	1.7 (0.80-4.8)	1.4 (0.50-3.0)	
PS1	0.63 (0.54-0.92)	2.5 (1.7-3.8)	-	
PS7F	1.5 (1.0-2.4)	4.3 (2.9-5.7)	-	
PS8	0.67 (0.36-1.3)	6.6 (3.5-13.6)	-	
PS9N	0.34 (0.15-0.84)	1.7 (1.2-2.3)	-	
PS12F	0.40 (0.33-0.54)	1.0 (0.65-1.3)	-	
PS19A	1.6 (0.70-4.3)	5.3 (2.4-9.1)	-	
PS4	0.10 (0.07-0.17)	3.7 (1.3-12)	1.0 (0.36-2.2)	
PS6B	0.13 (0.04-0.30)	3.1 (1.1-6.7)	1.6 (0.39-4.9)	
PS9V	0.11 (0.09-0.18)	2.1 (0.95-5.4)	2.6 (0.68-13)	
PS14	0.89 (0.34-0.83)	14 (4.7-44)	6.0 (2.6-13)	
PS18C	0.15 (0.09-0.21)	4.0 (2.6-11)	3.8 (1.9-5.2)	
PS19F	0.12 (0.05-0.21)	2.8 (1.2-4.8)	4.0 (2.0-11)	
PS23F	0.28 (0.15-0.66)	7.9 (2.3-21)	1.7 (0.93-2.7)	

GMT: Geometric mean antibody titer (µg/ml), 25th and 75th percentile between brackets; PS: pneumococcal serotype; PPV23-only serotypes (bold) and PPV23/PCV7 serotypes (*Italic)*. * = significant ($p<0.05$) difference GMT DS group versus healthy adults or children with recurrent infections (statistics for PCV7 serotypes only applied for children with recurrent infections using the same vaccination scheme).

Anti-pneumococcal IgG titers: DS subjects

Post-vaccination anti-pneumococcal IgG-antibodies were significantly higher ($p<0.001$) than the pre-vaccination IgG-antibodies in all DS subjects.

T-cell independent (TI) antibody responses (PPV-23-only)

67% (12/18) of the DS subjects reached ≥1.0µg/ml of anti-pneumococcal IgG-antibodies in response to serotype 3, a highly immunogenic serotype. All DS subjects reached ≥1.0µg/ml post-vaccination anti-pneumococcal IgG-antibodies in response to serotypes 1, 7F, 8, and 19A; 83% reached this value for serotype 9N and 39% for serotype 12F.

	Children with recurrent infections (N=17)	Children with recurrent infections [21] (N=25)	Healthy adults [19] (N=75)	Healthy elderly [20] (N=58)
	1x PPV-23	2x PCV-7 and 1x PPV-23	1x PPV-23	1x PPV-23
	Post-GMT	Post-GMT	Post-GMT	Post-GMT
	1.7 (0.91-3.9)	1.8 (0.67-3.1)	2.1 (1.2-4.1)	1.2 (0.60-4.6)
	3.0 (2.0-5.9)	0.33 (0.10-0.94)*	2.5 (1.3-5.0)	2.4 (0.95-6.3)
	3.8 (1.9-7.1)	0.82 (0.25-2.0)*	6.5 (3.7-14)*	19 (3.2-19)*
	8.7 (7.7-15)	-	7.1 (3.1-10)	2.9 (1.7-9.2)
	3.4 (2.5-8.0)*	-	4.8 (2.3-10)*	10 (2.9-11)*
	1.9 (1.2-3.7)*	-	1.2 (0.70-2.2)	1.9 (1.0-5.8)*
	4.5 (2.7-7.1)	0.89 (0.27-1.4)*	3.8 (2.1-9.2)	9.2 (6.4-9.2)
	2.9 (1.7-6.8)	2.3 (0.85-7.8)	3.3 (1.4-6.6)	1.9 (0.66-5.3)
	1.2 (0.63-2.6)	1.5 (0.69-4.6)	3.9 (1.8-10)	6.8 (1.8-17)
	1.6 (0.76-5.2)	1.7 (0.95-4.7)	2.8 (1.4-7.7)	8.4 (1.9-9.0)
	3.2 (0.69-14)	3.4 (1.7-8.8)*	11 (2.5-20)	20 (5.5-20)
	2.8 (1.8-5.1)	2.9 (1.8-7.9)	3.3 (1.1-8.7)	11 (4.3-12)
	2.5 (1.1-9.4)	4.4 (2.0-9.4)	6.5 (2.6-12)	8.4 (2.6-12)
	1.1 (0.62-2.2)	2.6 (1.3-5.9)*	2.5 (1.0-6.3)	3.8 (1.5-6.4)

T-cell independent antibody responses in DS subjects for PPV-23-only serotypes (serotypes 1, 3, 7F, 8, 9N, 12F and 19A) were compared with healthy adults and elderly, and children with recurrent infections without serious primary immunodeficiency. The DS subjects showed a similar post-vaccination response to serotype 3 as healthy individuals and children with recurrent infections (Table 2, no significant difference). In comparison to healthy individuals [19,20], DS subjects showed a similar anti-pneumococcal IgG response to serotypes 1, 8, and 19A, but a lower response to serotypes 7F, 9N and 12F (Table 2). DS subjects had a significantly lower post-vaccination response to serotype 9N and 12F ($p=0.024$ and $p=0.048$, respectively) compared to

Table 3 Percentage of response ≥1.0µg/ml for each serotype by patient category.

	Down syndrome (n=18)	Children with recurrent infections (n=17)	Healthy adults (n=15)	Children with recurrent infections (n=25) [21]
Vaccination scheme	**2x PCV-7 and 1x PPV-23**	**1x PPV-23**	**1x PPV-23**	**2x PCV-7 and 1x PPV-23**
PS1	100%	88%*	-	25%
PS3	67%	65%	60%	64%
PS7F	100%	86%	-	45%
PS8	100%	93%	-	-
PS9N	83%	86%	-	-
PS12F	39%	86%	-	-
PS19A	100%	86%	-	36%
PS4	83%	88%	53%	76%
PS6B	78%	65%	53%	68%
PS9V	72%	76%	67%	76%
PS14	100%	71%	93%	84%
PS18C	83%	94%	93%	84%
PS19F	78%	82%	87%	92%
PS23F	78%	71%	71%	79%

PS: pneumococcal serotype; PPV23-only serotypes (bold) and PPV23/PCV7 serotypes (*Italic).*

children with recurrent infections from the same laboratory. In comparison to English children with recurrent infections [21], DS subjects showed a significantly higher post-vaccination response to serotype 1, 7F and 19A ($p<0.001$, $p=0.003$ and $p=0.019$, respectively; original data kindly provided by R. Borrow).

T-cell dependent (TD) responses

61% and 89% of the DS subjects reached post-vaccination anti-pneumococcal IgG ≥1.0µg/ml to ≥6 and to ≥4 PCV-7-serotypes, respectively. T-cell dependent antibody responses in DS subjects for PCV-7 serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) were compared with English children [21] undergoing the same vaccination scheme (Table 2, Figure 1; original data kindly provided by R. Borrow). DS subjects showed comparable responses to all PCV-7 serotypes (no significant difference), except for serotype 14 and serotype 23F, which were significantly higher in the DS subjects (respectively $p=0.009$ and $p=0.049$).

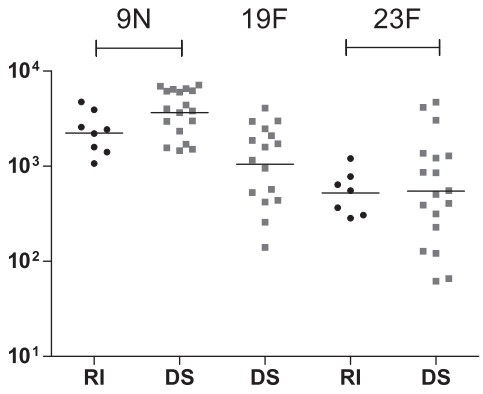

Figure 2 Opsonophagocytic assay.

Phagocytosis index (PI) post-vaccination to pneumoccal serotypes 9N, 19F and 23F; Geometric mean, logarithmic scale. DS = Down syndrome subjects (9N, 19F, 23F); RI = children with recurrent respiratory infections but without serious primary immunodeficiency (9N, 23F).

Opsonophagocytosis

OPA results for serotype 9N (PPV-23 only) were higher in the DS group compared to children with recurrent infections, but this difference was no longer significant after logarithmic transformation ($p = 0.058$). OPA results for serotype 23F (both PCV-7 and PPV-23) were comparable in both groups (no significant difference). No specific relation could be found between DS children suffering from recurrent respiratory infections and lower OPA titers to serotype 9N, 19F and 23F (Figure 2).

DISCUSSION

We found the titers of the anti-pneumococcal antibody response to be adequate for protection in the studied DS subjects. Their protein-conjugated T-lymphocyte-dependent combined PCV-7/PPV-23 responses were within the normal range. Despite the lower absolute B-lymphocyte count in DS [3,4], post-vaccination titers of T-lymphocyte-independent anti-polysaccharide PPV-23-only responses were not lower in comparison to healthy non-DS subjects. Functionality of the antibodies was adequate as well; the DS subjects responded with similar OPA titers to serotype 19F and 23F after repeated PCV7 or PCV13 vaccinations.

Why then do many DS subjects suffer from recurrent upper respiratory tract infections? And why do these recurrent infections subside after their first 6-8 years of

life? Well-known ear-nose-throat anatomical abnormalities in DS could be the cause for these respiratory infections [26,27,28]. However, these characteristic facial features remain the same with increasing age. An explanation could be that the humoral immune system in DS reaches an adequate maturation level after repeated antigen contact, as shown by us in this study, which protects against ongoing respiratory infections. Autoimmune diseases and hematological malignancies, on the other hand, increase with increasing age in DS [28]. Continuous infectious pressure on the DS immune system due to the intrinsic anatomical abnormalities could be a factor in the evolvement of this immunodysregulation: ongoing bacterial infections can lead to antibodies that cross-react with host tissue [29]. Then, with time the somatic hypermutation process, combined with selection by antigen, will result in high affinity antibodies, potentially also with specificity for autoantigens.

Our study is limited by the comparison of the results of our DS cohort with those obtained in other studies. However, in order to overcome potential differences we only included data obtained by the same multiplex bead based assay we used, because Borgers et al [19] showed that the serotype 3 titer does not show a good correlation between the multiplex bead-assay and the enzyme linked immunosorbent assay (ELISA) used in other studies.

The strength of our study is the inclusion of an opsonophagocytosis assay for affinity maturation after repeated priming. The repeated vaccination scheme we used showed production of high-affinity antibodies in the DS subjects comparable to healthy children [23], implying an adequate cooperation between B- and T-lymphocytes. In comparison, elderly subjects respond to pneumococcal vaccinations with lower affinity antibodies [22].

In conclusion, our DS subjects showed an adequate quantitative and qualitative immune response to PPV23 as well as PCV7 vaccines, clearly different from the profiles of immunosenescence or CVID. The DS subjects did not show signs of SPAD either [24,25].

Acknowledgements

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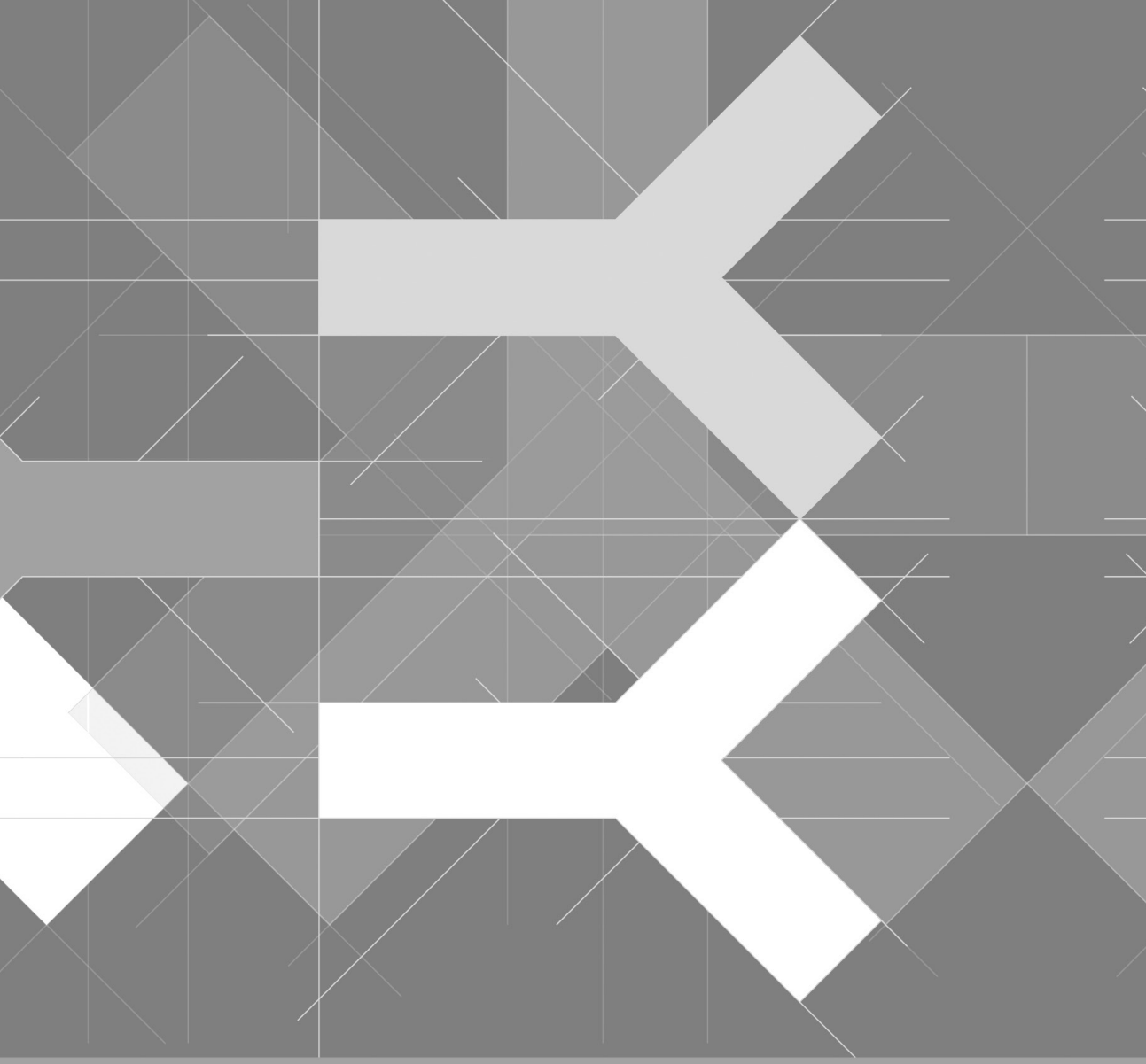

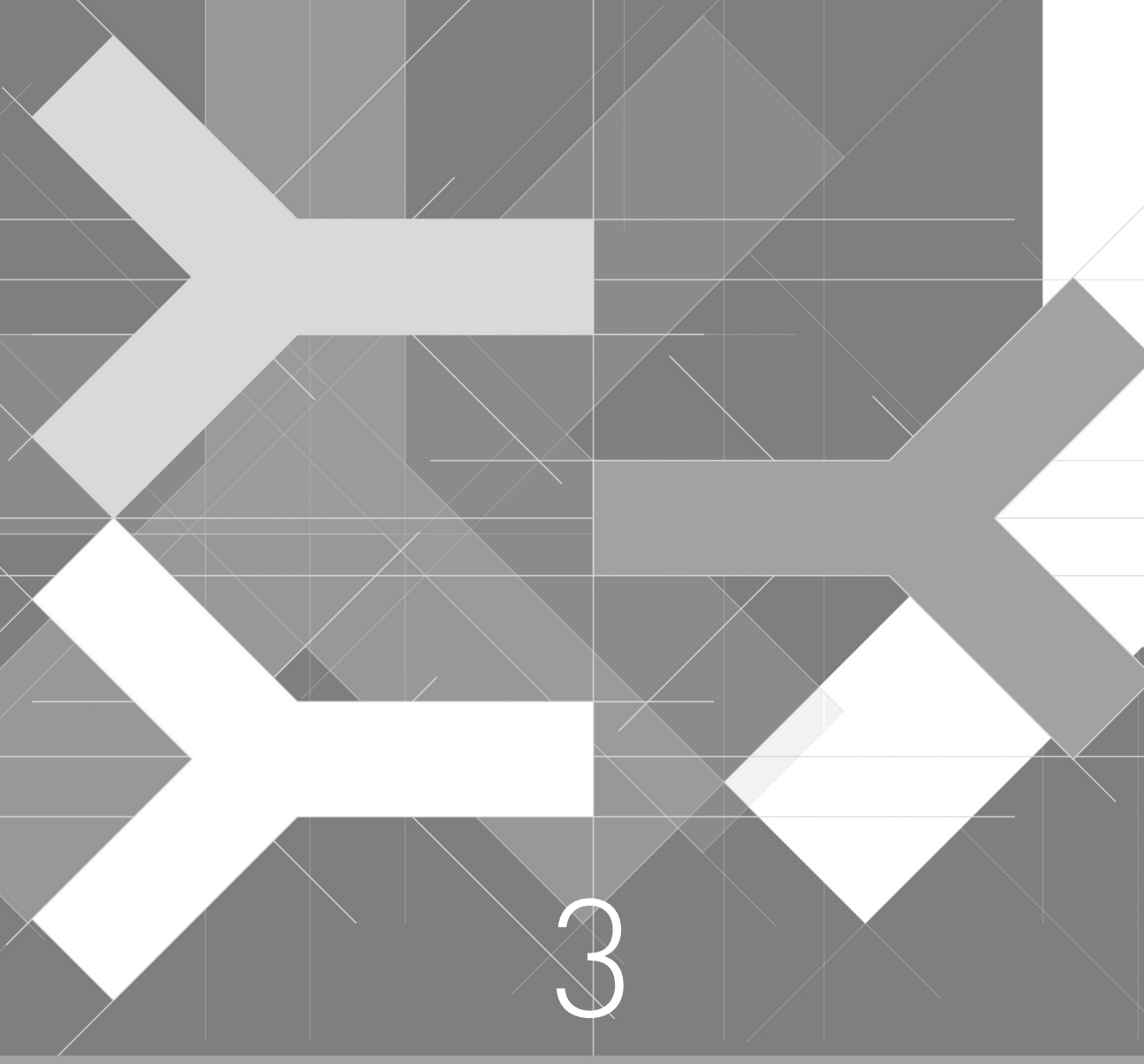
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SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Down syndrome (DS) is the most frequent cause of mental retardation in man.[1] The triad of increased incidence of infections, autoimmune diseases and haematological malignancies has led to the hypothesis of an altered adaptive immune system in Down syndrome.[2-7] During corrective heart surgery it was noticed that the macroscopic aspect of the DS thymus looked different.[8] As a result, immunological research in DS focused on the thymus and T-lymphocyte problems since the 1970s. Early occurrence of Alzheimer disease led to a hypothesis of precocious ageing in Down syndrome; until now, the observed clinical profile has been interpreted in the same context as premature immunosenescence of the thymus and T-lymphocytes. [9-12]

This thesis gives more insight in the qualitative and quantitative alterations found in the adaptive immune system of children with Down syndrome. In Part 1 the analysis of both T- and B-lymphocyte subpopulations and immunoglobulin (IgG, IgA, IgM) levels in different age groups in DS children is described. In Part 2 the quantity and quality of B- and T-lymphocyte responses to different types of antigen is investigated through vaccination studies, as models for T-lymphocyte dependent (TD) and T-lymphocyte independent (TI) immune responses.

T- and B-lymphocytes subpopulations in peripheral blood

In **chapter 4** we describe low naive T-lymphocyte counts in DS peripheral blood. Our study on T-lymphocyte subpopulations in Down syndrome shows that the normal expansion of naive helper-T (Th) and cytotoxic-T (Tc)-lymphocytes is lacking from the first years of life onwards. Especially naive helper-T-lymphocytes are decreased: e.g. in DS children aged 9-15 months median absolute numbers of naive Th are 0.91 x 10^9 cells/l as compared to 2.7 x 10^9 cells/l in age-matched control children (AMC).[13]

Decreased numbers of T-lymphocytes from an early age onwards can be the result of (partial) failure of thymic output of T-lymphocytes, (partial) failure of proliferation, increased apoptosis or a combination of these processes. Thymic anatomical alterations are well-known in DS and are already seen in DS fetuses. An impaired thymic output with reduced TREC counts in DS has been described.[14-17]

Thymic hypoplasia with reduced thymic output is an intrinsic feature of children with DiGeorge syndrome (DGS). These children show some similarities to DS children: they have a clinical picture of recurrent infections and higher incidence of autoimmune disease as well. In DGS, despite thymic hypoplasia, most subjects appear to gradually reach T-lymphocyte levels of healthy adults over time, and T-cell function seems relatively preserved in most cases in DGS.[18-20] Although the absolute numbers of cytotoxic-T-lymphocyte subpopulations in DS also approach age-matched control levels towards adulthood, low absolute counts of helper-T-lymphocytes continue to be present in all DS age groups.

Partial thymectomy during heart surgery in the first year of life can induce thymic hypoplasia and decrease thymic output. About fifty percent of DS children also suffer from congenital heart disease (CHD). However, non-DS children that undergo early CHD-surgery show a gradual recovery towards normal T-lymphocyte levels and normal function of T-lymphocytes.[21-23]

In chapter 4 we show that T-lymphocytes in Down syndrome children lack the proliferative expansion seen in normal children in their first years of life. Exposure to a diversity of antigens in the first years of life normally results in an enormous antigen-driven expansion of T- and B-lymphocytes in healthy children.[24-26] DS children often suffer prolonged and repeated respiratory infections due to their unfavorable upper and lower airway anatomy.[27] In a normal immune system, this would likely result in increased instead of decreased numbers of lymphocytes. It is likely that the DS T-lymphocytes eventually harbor a restricted repertoire, having shown such a profound lack of antigen-driven expansion in earlier years. Functional impairment in DS T-lymphocytes with decreased proliferative and antigen T-cell responses have been described which support this hypothesis.[15, 28-30] For instance, the in vitro proliferative response to phytohemagglutinin (PHA) is markedly below normal in DS infants as well as DS adults.[30-33] The clinical picture does not fit severe T-lymphocyte deficiency, however: DS subjects do not suffer from opportunistic

infections or severe failure to thrive. Our findings in chapter 4 do not support severe immunodeficiency either: cytomegalovirus (CMV)-seropositive DS children show similar absolute numbers (median 0.079 x 10^9 cells/l) of terminally differentiated ($CD27^-CD45RA^+$) cytotoxic-T-lymphocytes when compared to healthy children (median 0.067 x 10^9 cells/l), not increased absolute numbers as are described in children with primary CMV infections during immunosuppressive therapy (0.413 x 10^9 cells/l) or HIV-1 infection (0.369 x 10^9 cells/l). [34-36]

Enhanced cell death by apoptosis could play an extra role in DS besides decreased T-lymphocyte production and proliferation. Apoptosis data in DS is scarce and seems contradictive: increased telomere shortening is found in DS T-lymphocytes, which could lead to higher apoptosis rates. [37, 38] A recent study [39] however did not find increased apoptosis in peripheral T-lymphocytes, despite increased apoptosis markers on T-lymphocytes in earlier reports.[38, 40, 41]

So, decreased numbers and impaired functioning of T-lymphocytes in Down syndrome from an early age onwards seem to be the consequence of combined partial failure of thymic output and T-lymphocyte proliferation; apoptosis data is inconclusive.

In **chapter 5** we describe the B-lymphocyte subpopulations in the cohort of 95 DS children. We found that, apart from their T-lymphocyte alterations, children with Down syndrome show alterations in their B-lymphocyte subpopulations as well. Our data in chapter 5 show a profound B-lymphocytopenia in all DS age groups: e.g. the median absolute numbers of naive B-lymphocytes in the DS age group 9-15 months is 0.35 x 10^9 cells/l versus 1.07 x 10^9 cells/l in AMC; in the DS age group 15-24 months it is 0.17 x 10^9 cells/l versus 0.58 x 10^9 cells/l in AMC. DiGeorge syndrome subjects do not have an intrinsic B-cell defect next to their T-cell defect.[42] Recent studies support the hypothesis of an impaired B-lymphocyte production in the bone marrow with decreased B-lymphocyte output from birth in DS.[42, 43]

The majority of B-lymphocyte maturation and development in the periphery is T-cell dependent and takes place in the germinal centers. The most important B-lymphocyte product IgG is produced by $CD27^-IgG^+$ and $CD27^+IgG^+$ memory B-lymphocytes. It is most likely that $CD27^+IgG^+$ B-lymphocytes start out as $CD27^-IgG^+$ B-lymphocytes; $CD27^+IgG^+$ B-lymphocytes result from multiple consecutive germinal center reactions with increased proliferation and SHM levels. These B-lymphocytes show dominant use of IgG_1 and IgG_2 in comparison to $CD27^-IgG^+$ B-lymphocytes - produced in the primary GC reaction - with dominant use of IgG_1 and IgG_3. Apart from low naive B-lymphocytes, we describe a consistently decreased number of $CD27^+$-memory B-lymphocytes in all DS age groups. Despite this B-lymphocytopenia, DS children show a hypergammaglobulinemia with increased total IgG, IgG_1 and IgG_3 (but decreased IgM, IgG_2 and IgG_4 and normal IgA) serum levels from the age of three years onwards compared to healthy non-DS children in chapter 5. The hypergamma-

globulinemia found in DS is suggestive of dysregulation in class switching of B-lymphocytes within the germinal centers. It might be that memory B-lymphocyte proliferation is skewed with a preference towards B-lymphocytes with dominant use of IgG_1 and IgG_3 in primary and secondary germinal center reactions in DS.

We also found decreased absolute and relative numbers of $CD21^{high}$ and $CD23^+$-B-lymphocytes in DS. CD23 is a ligand of CD21; together they stimulate B-lymphocyte proliferation and differentiation. CD21 is the complement type 2 receptor; it has a role in the response to polysaccharide antigens like pneumococcal capsular elements. Median absolute numbers of $CD23^+$- and $CD21^{high}$-B-lymphocytes in DS are around one-third lower in all age groups. The highest expression of CD21 on B-lymphocytes is normally found in the splenic marginal zone ($CD27^+IgM^+IgD^+$) on natural effector B-lymphocytes. Natural effector B-lymphocytes play an important role in the prevention of sepsis by (polysaccharide capsuled) bacteria through T-cell independent processes.[44] DS children have higher morbidity and mortality of bacterial sepsis in comparison to age-matched individuals in their first years of life.[45] Further research on B-cell development needs to be performed to answer the question whether natural effector B-lymphocytes are indeed affected in DS.

The B-lymphocyte proliferation and differentiation and immunoglobulin-profile in DS is different from that in young non-DS children with recurrent respiratory tract infections. In them, repeated antigen responses lead to an increased pool of B-memory subpopulations resulting from multiple consecutive germinal center reactions.[46] These children often have low IgA and IgG_2 serum levels, but do not have B-lymphocytopenia or hypergammaglobulinemia.[47] Most of these children appear to have a slower peripheral B-cell maturation, without an apparent immunodeficiency after infancy.

The question remains whether decreased and altered B-lymphocyte subpopulations in combination with a hypergammaglobulinemia in Down syndrome are a true reflection of intrinsic B-cell defects. Another explanation would be a disturbed helper-T-lymphocyte interaction leading to a skewed control of peripheral B-lymphocyte maturation and development through impaired SHM and class switching.

Vaccination responses

The distribution of B-lymphocyte subpopulations in Down syndrome is reminiscent of the situation found in a subgroup of common variable immunodeficiency disorder (CVID) patients who suffer from recurrent infections and autoimmune diseases as well. CVID is a heterogeneous group of B cell disorders in combination with decreased Igs and deficient antibody response to protein (T-cell dependent) and polysaccharide (T-cell independent) antigens.[48-51] Different types of vaccination were used in this thesis as a model to study these different T-cell immune response pathways in order to get more insight into T- and B-lymphocyte functional capacity and communication

in DS, in comparison with different patient groups with well-known T- or B-lymphocyte defects.

In **chapter 6** antibody levels and avidity were tested in 22 DS children after TT booster vaccination at 4 and 9 years of age. Non-DS children with decreased numbers of helper-T-lymphocytes such as children with DiGeorge syndrome [52] or HIV-1 infection [53, 54] mount a protective response but with lower mean IgG anti-TT-antibody titers. DS children produced protective anti-TT antibody titers in both age groups. However, post-booster vaccination anti-TT-antibody titers and IgG_1-avidity (most dominant IgG produced after TT) in the 4-year-old children with DS were significantly lower. After booster vaccination at 9 years of age, DS children reached anti-TT-antibody total IgG within the adult reference range, but IgG_1-avidity was still significantly decreased. Lower post-TT avidity despite repeated booster doses suggests a subtle selection problem of memory B-lymphocytes within the germinal centers in DS. Such selection problems can occur through intrinsic B-lymphocyte defects or through an impaired interaction between Th and B-lymphocytes.

In **chapter 7** we determined the haemagglutination-inhibition (HI) titer after two doses of influenza A/H1N1 vaccination in 48 DS children. According to the WHO-definition of correlate for protection (HI titer ≥1:40), 92% of DS children reach protective levels after vaccination. However, this HI cut-off value has not been studied for influenza A/H1N1 vaccination in children. Pre-vaccination data from the available literature show that up to 30% of healthy children reach a HI titer of ≥1:40 without a known history of previous influenza A/H1N1 vaccination or active influenza A/H1N1 infection. The recently proposed new cut-off value to predict the conventional 50% clinical protection rate in children, ≥1:110[55], is reached in only 27% of our DS cases. If this HI cut-off value is applicable, our results would further support a T-lymphocyte or T-B-interaction problem in DS.

Others have described impaired immunity in terms of cytokine and antibody response in DS in research based upon protein-prototype vaccinations such as influenza, hepatitis B and tetanus as well.[56, 57]

In **chapter 8** MenC polysaccharide specific (MenC/PS) antibody titers after a single MenC vaccination were tested in 19 DS children. All DS children reached protective levels. However, in comparison to healthy adults MenC/PS antibody titers were lower, despite the fact that 9 DS children showed hypergammaglobulinemia. Our data show that protein conjugation does not fully overcome the impaired antibody production to this polysaccharide antigen in DS.

In **chapter 9** we determined anti-pneumococcal serotype antibody titers (quantitative test) and opsonophagocytosis (qualitative test) after a combined scheme of PCV7 (2x) and PPV23 (1x) in 18 DS subjects between 6 and 24 years of age. The results show adequate serotype-specific antibody titers when using both the WHO cut-off value (≥0.35µg/ml) as well as when using the higher cut-off value of

≥1.0μg/ml for prevention of mucosal infection in response to most conjugated and unconjugated serotypes tested. DS subjects showed lower responses to serotypes 7F, 9N and 12F in comparison to healthy individuals, however. Opsonophagocytosis activity as measured against pneumococcal serotypes 9N, 19F and 23F was normal. We conclude that these DS subjects do not have a defect in the anti-polysaccharide antibody response.

The adequate response to unconjugated pneumococcal serotypes requires mature B-lymphocytes and therefore argues against severe B-lymphocyte problems or specific anti-polysaccharide antibody deficiency (SPAD) [47] in Down syndrome subjects. The adequate antibody titer after TT-booster in combination with an adequate pneumococcal vaccination antibody response as seen in our Down syndrome subjects are clearly different from CVID patients, despite their apparent clinical resemblance with recurrent infections, auto-immune phenomena and malignancies, and immunological resemblance with altered memory B-lymphocyte subpopulations.

Overall, the vaccination studies performed in **part 2** show protective post-vaccination antibody levels (using current protective cut-off values) in DS, although the qualitative and quantitative antibody responses differ per vaccination and subject. The pattern of vaccination response in DS is not comparable with the pattern seen in severe T-, or B-lymphocyte defects, but subtle impairments of the selection process of memory B-lymphocytes within germinal centers of lymph nodes do seem to occur.

Immunosenescence?

With normal ageing, fewer B-lymphocytes are produced in the bone marrow, and thymic involution with low output of naive lymphocytes ensues.[58, 59] At first sight, the DS profile seems to fit precocious immunosenescence (chapter 2), because altered thymic anatomy and decreased naive B- and T-lymphocytes occur both in DS and normal ageing.

However, alterations of the T- and B-lymphocyte compartment in DS are present from the very beginning: newborns and fetuses [60] with DS already show an altered thymic anatomy with impaired thymic output [61] and lower TREC counts [43] as well as low naive B-lymphocytes[60] and lower kappa-deleting recombination excision circles (KREC) counts. [43]

Decreased output of naive B- and T-lymphocytes is mirrored in ageing individuals by an increase in effector and memory B, Th and Tc numbers. The lymphocyte pool fills up with specific oligoclonal memory T and B-lymphocytes, mainly memory helper (Th) T-lymphocytes, terminally differentiated cytotoxic (TD Tc) T-lymphocytes and more restricted TCR-γδ Tc and Th with less diversity.[62-65] The lymphocyte pool becomes more experienced but less flexible, and the cells show a more restricted repertoire and reduced proliferative response. We did not find an early shift towards these oligoclonal memory T-lymphocyte subsets (chapter 4) or an early expansion towards memo-

ry-B-lymphocytes (chapter 5). Serum immunoglobulin levels remain stable during normal ageing, but in DS a profound hypergammaglobulinemia develops from around 3 years of age onwards.

With normal ageing, decreased output and functional deficiency of the T- and B-lymphocyte pool hampers the adaptive immune response to both T-cell dependent and independent vaccinations.[58] Quantitative antibody responses become lower, decline faster and the affinity of the antibodies is diminished [58, 66-69], especially in response to polysaccharide vaccines. [66] Our data show that DS subjects respond with adequate opsonophagocytosis assay titers to serotype 19F and 23F after repeated conjugated pneumococcal vaccinations (chapter 9).

The clinical profile with higher rates of infections, malignancies and autoimmune disease occur both in DS and normal ageing.[70-73] However, the pattern is different: DS subjects mainly show higher frequencies of hypothyroidism[72], celiac disease[74] and diabetes mellitus type 1[75] as opposed to elderly subjects with increased occurrence of e.g. diabetes mellitus type 2 and rheumatoid arthritis.[76] Malignancies in DS are mainly haematological in contrast to elderly who show an increase in non-haematological malignancies as well.[77] So, immunosenescence does not seem to be an issue in DS.

Down syndrome: a syndromic immunodeficiency

Down syndrome is the most common chromosomal abnormality in humans. The prevalence of Down syndrome in the Netherlands is higher nowadays than during the 80s and 90s.[97] Early diagnosis and treatment of congenital heart defects has further decreased mortality in the first years of life in Down syndrome subjects.[98, 99] But despite all health care improvements, the clinical profile of infections, autoimmune diseases and haematological malignancies still causes high morbidity and mortality in Down syndrome.

In this thesis we show that it is unlikely that early immunosenescence explains the immunological alterations in DS. Parallels in clinical profile, antibody response to vaccination and T- and B-lymphocyte subpopulations between DS and specific immunodeficiencies such as DiGeorge syndrome (DGS) and common variable immunodeficiency disorders (CVID) exist, however, there are also obvious differences. We conclude that Down syndrome subjects have a unique profile of a mild combined intrinsic T- and B-cell immunodeficiency.

Awareness of and more research on this unique syndromic immunodeficiency is important for early recognition of immunodeficiency and immunodysregulation in Down syndrome, and for appropriate intervention in our day to day clinical practice.

FUTURE PERSPECTIVES

This thesis has provided more insight into the DS immune system, but many questions remain. Most studies performed in DS regarding lymphocyte subpopulations and function, including our own, are cross-sectional in nature. A large longitudinal cohort study with DS newborns and healthy non-DS controls would enable correlation of immune alterations with the clinical picture in DS.

Based on our data and current knowledge, we cannot determine whether the problem in communication between Th and B cells in DS is based upon inadequate Th help to B-lymphocytes or upon the incapacity of B-lymphocytes to respond to Th help, or both.[38, 78] Systematic analysis of antigen-presentation and processing by B-lymphocytes and of the processes influencing the choices made in somatic hypermutation and class switch recombination could shed further light on this issue. Recent new insights regarding the immunological synapse between antigen-presenting cells (APCs)[79] and B-lymphocytes and between B-Th[80] would be interesting to study in DS as well. Also, T-lymphocyte selection processes in the thymus can be influenced by defective or altered network connections within the immunological synapse between APC and thymocytes. Earlier reports found an increased expression of cell adhesion molecules in thymic epithelia and thymocytes[81], but with decreased T-lymphocyte adhesion to ICAM-1 in another study in DS subjects.[82]

The immune system has to walk a fine line to preserve the integrity of the body: it has to produce an adequate immune response to non-self without inducing immune reaction with damage to self. The higher frequencies of autoimmune diseases (e.g. celiac disease, diabetes mellitus, thyroid disease) and lymphoproliferation (e.g. leukemia) point toward immune dysregulation in DS.[83] Insight in the mechanism causing autoimmunity in DS is limited. To our current knowledge, environmental triggers are needed for the development of autoimmune disease. Especially infections have been implicated in the onset and promotion of autoimmunity.[84, 85] The continuous infectious pressure put on the DS immune system due to their anatomic and functional respiratory tract abnormalities could thus play a role. Non-DS publications highlight the increased incidence of autoimmunity in partial as opposed to severe T-cell immunodeficiencies. This might be caused by reduced effectiveness of communication between thymocytes and epithelial cells due to disordered thymic microarchitecture, altering the efficiency of central tolerance.[86] This central tolerance is achieved by a process called negative selection: T-cells with too high affinity to self-peptide presented on MHC undergo apoptosis in the thymus. These self-peptides are tissue-specific self-antigens from different parts of the human body, their expression on thymic epithelial cells is controlled by AIRE (autoimmune regulator). A recent study on the thymus transcriptome showed significant hypoexpression of more than 400 genes related to cell division and immunity in young DS subjects

including AIRE.[87] Lack of gaining adequate central tolerance can result in multiple organ-specific autoimmune disorders, but it does not lead to an increased allergy rate. Indeed, DS subjects have increased organ-specific autoimmune disorders, and a decreased allergy rate. So, it is tempting to speculate that thymic hypofunction with altered T-lymphocyte development and interaction (including inadequate central tolerance) leads to autoimmunity in DS.

From a clinical perspective, it is interesting to speculate whether aggressive treatment and/or prevention (e.g. ENT interventions, vaccinations, antibiotics) of respiratory tract infections in the first years of life will positively influence the development of the DS immune system. But also whether decrease of respiratory tract infections can lead to improvements in cognitive, motor and speech development [102], achieving a higher quality of life for children with DS.

Genetic predisposition can influence the sensitivity for actual development of autoimmune diseases and malignancies. Genetic studies on DS provide interesting insight. DS is not a monogenic disorder, but a consequence of an extra (critical part of) chromosome 21.[88] However, contrary to common belief of 150% genetic overexpression of chromosome 21 in all DS organs, different expression of chromosome 21 genes has been described depending on tissue and cell type.[89]

Besides, genome-wide expression analysis in DS revealed that hundreds of genes related to immune function *not* located on chromosome 21 appear to be dysregulated as well, demonstrating the pervasive effects of trisomy 21 on the whole genome. [90-93] A typical example from earlier publications are somatic mutations in the GATA1 gene (X-chromosome) leading to transient leukemoid reactions in the neonatal period in Down syndrome.[94, 95] Somatic mutations in the JAK2 gene (chrosomome 9) are associated with acute lymphoblastic leukemia in DS. Additional complexities may exist due to epigenetic changes that may act differently for DS in general and for specific organs.[96] These genetic studies further support our hypothesis that DS subjects suffer from a unique syndromic immunodeficiency.

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11

NEDERLANDSE SAMENVATTING

Dit proefschrift beschrijft het onderzoek dat gedaan is naar het afweersysteem bij kinderen met downsyndroom. Downsyndroom is de meest voorkomende chromosomale afwijking; het wordt veroorzaakt door een extra – 3e – chromosoom 21 (de zogenaamde 'trisomie' 21). Ieder jaar worden er in Nederland circa 245 kinderen met downsyndroom geboren. Zij worden vaak begeleid door een vast team van hulpverleners op speciale Down-poliklinieken volgens een landelijk protocol.

Kinderen met downsyndroom hebben typische uiterlijke kenmerken met onder andere een platte neusbrug, epicanthus plooien (opvallende plooien langs de binnenste ooghoeken), een uitstekende tong, kleine oren en een korte nek. Zij hebben vaak een viervingerhandlijn en een sandal gap (extra grote ruimte tussen de grote teen en de tweede teen). Daarnaast hebben zij een kleine lengte, verlaagde spierspanning (hypotonie) en een verschillende mate van ontwikkelingsachterstand (mentale retardatie). Ongeveer 50% van de kinderen met downsyndroom heeft een aangeboren hartafwijking. Kinderen met downsyndroom hebben een andere anatomie van het KNO-gebied: bijvoorbeeld een vernauwde gehoorgang en een kleinere en anders aangelegde buis van Eustachius. Kinderen met downsyndroom hebben meer speekselvloed, last van zuurbranden (gastro-oesofagale reflux) en ook in de onderste luchtwegen kunnen anatomische veranderingen aanwezig zijn.

Met name in de eerste levensjaren hebben kinderen met downsyndroom veel luchtweginfecties. Complicaties door luchtweginfecties zijn de belangrijkste oorzaak van overlijden in alle leeftijdsgroepen bij downsyndroom, ondanks alle verbeteringen in de zorg voor deze groep. De veranderde anatomie van het KNO-gebied en de lagere luchtwegen, een lagere spierspanning en gastro-oesofagale reflux met microaspiraties worden vaak als oorzaak voor de verhoogde infectiegevoeligheid aangewezen. Maar afwijkingen in het afweersysteem zouden ook kunnen bijdragen aan het optreden van (ernstige) infecties. Als kinderen met downsyndroom ouder worden zie je dat andere aandoeningen, zoals autoimmuunziekten en leukemie, vaker voorkomen. Bij autoimmuunziekten valt het lichaam de eigen cellen aan. Voorbeelden zijn suikerziekte (diabetes mellitus type 1, DM1), glutenintolerantie (coeliakie) en schildklierproblemen. Bij leukemie ontaarden gewone bloedcellen kwaadaardig. Dit kan wijzen op een ontregeld afweersysteem omdat normaliter het afweersysteem dit ondervangt. Er lijkt bij downsyndroom sprake te zijn van een combinatie van meer infectieproblemen en daarnaast van immuundysregulatie met meer autoimmuunziekten en leukemie. Deze trias wordt ook gezien bij mensen met bekende aangeboren afweerziekten en ook bij normale veroudering (waarbij het afweersysteem ook minder gaat functioneren).

In het verleden is al onderzoek gedaan naar het afweersysteem bij downsyndroom. Omdat ongeveer de helft van de kinderen met downsyndroom een hartafwijking heeft, worden veel jonge kinderen met downsyndroom in het eerste levensjaar geopereerd. Tijdens deze operaties viel het op dat de thymus – het belangrijkste

afweerorgaan voor T-lymfocyten productie - er anders uit zag. Bij downsyndroom is daarom in het verleden met name verder gekeken naar de afwijkingen die werden gevonden in de thymus en in de T-lymfocyten. De afwijkende anatomie van de thymus in combinatie met de veranderingen binnen de T-lymfocyten, naast het vaker voorkomen van dementie op jonge leeftijd leken het beste te passen bij vervroegde veroudering. Er werd geconcludeerd dat de veranderingen die bij downsyndroom in het afweersysteem werden gevonden het directe gevolg van die vervroegde veroudering waren. Inmiddels zijn de onderzoekstechnieken verder ontwikkeld en zijn er nieuwe inzichten in hoe het afweersysteem werkt. We hebben daarom opnieuw onderzoek verricht naar T-lymfocyten en hebben ook de B-lymfocyten en de kwaliteit van de afweerreactie bij kinderen met downsyndroom in kaart gebracht. Onze vraag was hoe het verworven afweersysteem bij downsyndroom is opgebouwd en of dit leidt tot een afweerprobleem dat vergelijkbaar is met bekende aangeboren afweerstoornissen. Verder willen we weten of de eerder voorgestelde theorie aangaande vervroegde veroudering van het afweersysteem bij kinderen met downsyndroom klopt.

Bij normale veroudering van het verworven afweersysteem verwacht je verminderde aanmaak van nieuwe B- en T-lymfocyten in respectievelijk het beenmerg en de thymus. De productie van naïeve lymfocyten neemt af met het ouder worden en eerdere generaties zijn grotendeels uitgerijpt tot effector en geheugenpopulaties met een beperkt repertoire. Het systeem kan zich niet meer zo flexibel aanpassen en vermeerderen in reactie op het binnendringen van (nieuwe) ziekteverwekkers. De antistoffen geproduceerd door B-lymfocyten wijzigen met de leeftijd niet qua hoeveelheid, maar ze zijn wel vaak van een slechtere kwaliteit. Dit wordt veroorzaakt door het beperktere repertoire van de B-lymfocyten als gevolg van eenzijdige (klonale) uitgroei en minder goede hulp van helper-T-lymfocyten. Dit leidt tot een slechtere antistof-respons op vaccinaties en een toename van infecties, maligniteiten en auto-immuunziekten met het rijpen der jaren.

Het promotieonderzoek bestaat uit twee delen: in **Deel 1** wordt gekeken naar de samenstelling van het verworven afweersysteem bij downsyndroom: de B-lymfocyten en de T-lymfocyten. **Deel 2** van het proefschrift gaat over de functionele kwaliteit van het verworven afweersysteem bij downsyndroom, getest door middel van vaccinatie en de reactie hierop (vaccinatierespons). De resultaten in deze thesis nopen ons vraagtekens te zetten bij de hypothese van vervroegde veroudering van het verworven afweersysteem bij downsyndroom.

RESULTATEN PROEFSCHRIFT

Deel 1: lymfocytensubpopulaties

In **hoofdstuk 2** wordt als inleiding een overzicht gegeven van eerdere publicaties en hypotheses over het afweersysteem bij downsyndroom. In **hoofdstuk 3** wordt het verworven afweersysteem bij downsyndroom vergeleken met het verworven afweersysteem bij normale veroudering en bij zeldzame ziektes waarvan bekend is dat er sprake is van vervroegde veroudering. Bij downsyndroom wordt al vanaf de geboorte een afwijkende anatomie van de thymus gezien en lagere absolute aantallen naïeve T- en B-lymfocyten met lagere aantallen nieuwe T-lymfocyten die recent de thymus hebben verlaten. Er wordt geen antigeen-gedreven uitbreiding gezien van effector- en geheugen-lymfocyten met afname van de naïeve lymfocyten, zoals bij veroudering. Het klinische beeld van toegenomen maligniteiten, autoimmuunziekten en infecties bij downsyndroom lijkt weliswaar op dat van ouderen, maar er zijn wel duidelijke verschillen. Bij downsyndroom worden vooral auto-immuunziekten zoals coeliakie en DM1 gezien en maligniteiten zoals acute lymfatische en myeloïde leukemie, een patroon dat duidelijk anders is dan bij (vervroegde) veroudering.

In **hoofdstuk 4** zijn de T-lymfocytensubpopulaties bestudeerd. Vijfennegentig kinderen met downsyndroom werden geïncludeerd en vergeleken met leeftijdsgenoten. Zowel helper-T als cytotoxische T-lymfocyten zijn verlaagd aanwezig vanaf de geboorte bij downsyndroom. De enorme expansie van naïeve T-lymfocyten die bij kinderen zonder downsyndroom wordt gezien, zien we niet terug in ons cohort van kinderen met downsyndroom; vermoedelijk blijft het repertoire van de cellen door de verminderd doorgemaakte expansie beperkt. De absolute aantallen cytotoxische-T-lymfocyten laten een herstel zien richting leeftijd gerelateerde referentiewaarden na de eerste levensjaren, maar dit wordt vooral veroorzaakt doordat de expansie afneemt bij de leeftijdsgenoten. De aantallen helper-T-lymfocyten blijven laag. Dit alles wijst op een aangeboren anders functioneren van het T-lymfocytaire compartiment bij downsyndroom.

Door herhaald contact met antigenen rijpen de aanwezige T-lymfocyten verder uit, waardoor het afweersysteem minder flexibel wordt in het maken van een adequate afweerreactie op nieuwe bacteriën en virussen. Bij het ouder worden is dat duidelijk te zien: helper-T-geheugenlymfocyten, terminaal gedifferentieerde (TD) cytotoxische-T-lymfocyten en T-lymfocyten met nog weinig flexibiliteit voor aanpassing van de T-cel afhankelijke afweerreactie krijgen de overhand. Bij kinderen met downsyndroom vonden we juist geen verhoogde aantallen van deze subpopulaties, hetgeen tegen de hypothese van vervroegde veroudering van het afweersysteem bij downsyndroom pleit.

Het cytomegalievirus (CMV) is een virus dat cytotoxische-T-lymfocyten activeert en uit laat rijpen richting terminaal gedifferentieerde cytotoxische-T-lymfocyten. Het

aantal TD cytotoxische-T-lymfocyten dat bij contact met CMV wordt aangemaakt is een maat voor de kwaliteit van de T-lymfocyten functie, zo blijkt uit eerder onderzoek. Patiënten met een infectie met humaan immunodeficiëntie virus (HIV) maken bijvoorbeeld in reactie op CMV veel meer TD cytotoxische-T-lymfocyten aan dan gezonde mensen. Bij de kinderen die bewezen contact hadden gehad met CMV in onze groep met downsyndroom vonden we absolute aantallen TD cytotoxische-T-lymfocyten die vergelijkbaar waren met gezonde kinderen en lagere aantallen dan bij kinderen met HIV of kinderen met afweer-onderdrukkende medicatie. Deze resultaten wijzen erop dat er hooguit een milde stoornis in de functie van T-lymfocyten is bij downsyndroom.

In **hoofdstuk 5** worden de B-lymfocytensubpopulaties en immuunglobulinen (IgG, IgA, IgM; IgG-subklassen) bij de 95 kinderen met downsyndroom beschreven. De meest opvallende bevinding is dat de B-lymfocyten verlaagd zijn in alle leeftijdscategorieën. Ondanks deze persisterende B-lymfocytopenie wordt juist een toename van de immuunglobulinenproductie gezien. Vanaf de leeftijd van drie jaar is sprake van verhoogde immuunglobulinesubklassen IgG_1 en IgG_3 met verlaagde waarden van IgG_2 en IgG_4. Bij veroudering verandert de hoeveelheid immuunglobulinen doorgaans niet, maar de kwaliteit neemt wel af.

Daarnaast vonden wij een afwijkende verdeling van de perifere B-lymfocytensubpopulaties. Er zijn verlaagde aantallen $CD27^+$, $CD21^{high}$ en $CD23^+$ B-lymfocyten. Dit patroon wordt ook gezien bij een subgroep van patiënten met de afweerstoornis CVID (common variable immunodeficiency). Bij CVID is sprake van een verlaagd IgG in combinatie met een verlaagd IgA en/of IgM en een gestoorde antistofrespons. De patiënten hebben last van recidiverende luchtweginfecties met vaak permanente schade aan de lagere luchtwegen tot gevolg, en een verhoogde kans op maligniteiten en autoimmuunziekten. Hoewel dit lijkt op het klinische beeld bij downsyndroom is bij CVID juist sprake van verlaagde immuunglobulinen en niet van verhoogde immuunglobulinen zoals bij downsyndroom.

Deel 2: vaccinatieresponsen

Het verworven afweersysteem gebruikt twee verschillende routes om een adequate afweerrespons te maken tegen antigenen, de T-cel afhankelijke en T-cel onafhankelijke route. In dit proefschrift zijn vaccinaties als model gebruikt om deze verschillende routes van het verworven afweersysteem te testen.

Met de eiwit-antigenen tetanus toxoid en influenza A/H1N1 ("Mexicaanse griep") wordt de T-cel afhankelijke route getest. Bij herhaaldelijke blootstelling aan eenzelfde antigeen zoals een herhaalde vaccinatie met tetanus toxoid-vaccin, verwacht je activatie van geselecteerde geheugen B-lymfocyten die antistoffen met hoge aviditeit (bindingskracht) produceren. Kinderen zonder downsyndroom die een probleem hebben in hun T-lymfocyten (bijvoorbeeld kinderen met HIV infectie of DiGeorge

syndroom) laten een verminderde hoeveelheid antistofproductie zien na herhaalde tetanus toxoid-vaccinatie en na Mexicaanse griep-vaccinatie. In geval van ernstige T-lymfocytstoornissen zie je zelfs een afwezige antistofrespons, maar dat is bij downsyndroom niet het geval.

Met de pneumokokken-vaccinatie Pneumo-23, test je de T-cel onafhankelijke route. Dit vaccin bestaat uit polysachariden (suikermoleculen) zonder eiwit-antigenen. Hiervoor zijn ervaren, meer gerijpte B-lymfocyten nodig omdat ze geen hulp kunnen krijgen van T-lymfocyten. Bij ernstige B-lymfocytdefecten zie je dan ook een afwezige antistofrespons tegen polysachariden. Bij CVID is zowel de antistofrespons na zo'n pneumokokkenvaccinatie als na herhaalde tetanus toxoid-vaccinatie verlaagd. Met meningokokken serotype C (MenC) vaccinatie en met de heptavalente pneumokokken-vaccinatie (Prevenar) wordt de respons op een eiwit-suiker combinatie middels de T-cel afhankelijke route getest. Door een stuk eiwit (conjugaat) toe te voegen aan een polysacharide (suiker)- vaccin krijgt de B-lymfocyt toch weer hulp van de helper-T-lymfocyt, mits de B-lymfocyt het stuk eiwit op de juiste wijze kan verwerken en presenteren aan de helper-T-lymfocyt.

In **hoofdstuk 6** worden de hoeveelheid antistoffen en hun aviditeit (bindingskracht) na herhaalde tetanus toxoid-vaccinatie getest bij 22 kinderen met downsyndroom rond de leeftijd van 4 en 9 jaar conform het Nederlandse Rijksvaccinatieprogramma. Bij kinderen met downsyndroom wordt bij 4 en 9 jaar een beschermende antistoftiter gevonden. Bij 4 jaar is de hoeveelheid antistoffen (IgG en IgG-subklassen) lager dan bij kinderen zonder downsyndroom, bij 9 jaar is de hoeveelheid antistoffen gelijk. De hoeveelheid antistof verbetert dus bij herhaalde vaccinatie met tetanus toxoid. Desondanks is de bindingskracht van deze antistoffen bij 9 jaar nog steeds lager bij kinderen met downsyndroom.

In **hoofdstuk 7** wordt gekeken naar de hemaglutinatie-inhibitie (HI-) titer na twee doses van influenza A/H1N1 vaccinatie bij 48 kinderen met downsyndroom. De oude WHO-definitie van bescherming (namelijk het halen van een HI-titer van ≥1:40) wordt door 92% van de gevaccineerde kinderen met downsyndroom gehaald, een nieuw voorgesteld afkappunt (nl HI-titer van ≥1:110) voor gezonde kinderen wordt door slechts 27% van de kinderen met downsyndroom gehaald. Bij een ernstig T-lymfocytendefect zou je een falende antistof-respons verwachten. De kinderen met downsyndroom laten zien dat ze wel op dit vaccin kunnen reageren, maar met een lagere antistof-respons.

In **hoofdstuk 8** wordt bij 19 kinderen met downsyndroom na eenmalige geconjugeerde meningococcen C (MenC-) vaccinatie gekeken naar de specifieke antistof-titers. De antistofrespons na MenC-vaccinatie was adequaat bij de geteste groep met downsyndroom, maar lijkt wel minder lang stand te houden. Of dit klinische consequenties heeft is niet duidelijk omdat cijfers over de incidentie van hersenvliesontsteking en de oorzakelijke ziekteverwekkers specifiek voor downsyndroom ontbreken.

In **hoofdstuk 9** wordt gekeken naar de specifieke antistof-respons na een gecombineerd schema van tweemaal geconjugeerd (Prevenar, T-cel afhankelijk) en eenmaal ongeconjugeerd (Pneumo-23, T-cel onafhankelijke afweerreactie) pneumokokkenvaccin bij 18 individuen met downsyndroom ouder dan 5 jaar. Hiervoor is de hoogte van antistoffen tegen verschillende pneumokokken-serotypen (stukken suikerkapsel) uit de vaccinaties gemeten. Daarnaast werd een zogenaamde opsonisatiefagocytose test gedaan voor 3 pneumokokken-serotypes. Dit is een test die de kwaliteit van de antistoffen kan meten, dat wil zeggen hoe goed de antistoffen vastplakken aan de stukken pneumokok en er voor kunnen zorgen dat andere witte bloedcellen (fagocyten) de pneumokok kunnen herkennen en kunnen doden. De groep met downsyndroom reageert adequaat op de geteste geconjugeerde en ongeconjugeerde pneumokokken-serotypes, zowel qua specifieke antistoftiters als qua opsonisatie. De adequate antistofrespons na ongeconjugeerde pneumokokkenvaccinatie bij downsyndroom past niet bij een ernstig primair B-lymfocytendefect, omdat zij voor deze T-cel onafhankelijke afweerreactie blijkbaar voldoende ontwikkelde B-lymfocyten hebben. Ouderen hebben vaak een slechtere afweerrespons op pneumokokken-vaccinaties met lagere antistofkwaliteit. De afweerrespons bij downsyndroom is echter wel goed en niet vergelijkbaar met die van ouderen. In combinatie met voldoende antistofrespons na tetanus toxoid-vaccinatie is de vaccinatie-respons bij downsyndroom ook niet vergelijkbaar met die bij CVID patiënten.

De uitgevoerde vaccinatie-studies laten zien dat veelal een zogenaamde 'beschermende antistoftiter' wordt opgebouwd, maar dat de kwaliteit en kwantiteit van de antistoffen wel wisselend is.

CONCLUSIE

Bij downsyndroom is het verworven afweersysteem al vanaf de geboorte aangedaan. Kinderen met downsyndroom beginnen al met lagere aantallen B- en T-lymfocyten. Het gebrek aan cellen wordt niet opgevuld met bepaalde geheugencellen zoals je ziet bij veroudering. Er treed geen goed herstel van de aantallen B- en Th-lymfocyten op. Onze resultaten passen niet bij de theorie van vervroegde veroudering van het afweersysteem bij downsyndroom. In combinatie met het klinische beeld van frequente infecties lijkt dit het meest in de richting te wijzen van een intrinsiek afweerprobleem. Het meer voorkomen van autoimmuunziekten en leukemie in combinatie met de verhoogde immuunglobulinen vanaf de leeftijd van drie jaar suggereert tevens immuundysregulatie.

Het patroon van afweerreacties na de verschillende typen van vaccinaties bij kinderen met downsyndroom is niet vergelijkbaar met die van vervroegde veroudering van het afweersysteem. Ook is er geen sprake van een ernstig B- of T-lymfocyten defect. Toch zijn er wel subtiele problemen in de selectie en uitgroei van de juiste

B-lymfocyten waardoor je soms lagere of minder goede antistoffen krijgt na de T-cel afhankelijke vaccinaties. Op basis van de gevonden verschillen in vaccinatierespons lijkt er een, weliswaar mild, probleem te zijn in de samenwerking en communicatie tussen de B- en T-lymfocyten. Bij welk van de twee de oorzaak van de slechtere samenwerking moet worden gezocht, is nog niet duidelijk.

Dit leidt tot een nieuwe hypothese: mensen met downsyndroom hebben een intrinsieke afweerstoornis met een gecombineerd mild T- en B-lymfocyten probleem. Dit afweerprofiel is niet vergelijkbaar met bekende afweerstoornissen of versnelde veroudering, maar uniek voor downsyndroom.

Niet alle mensen met downsyndroom hebben evenveel last van deze afweerproblemen. Maar downsyndroom is wel de meest voorkomende chromosomale aandoening in Nederland en de trends van de afgelopen jaren wijzen ook niet op een daling van het aantal pasgeborenen met downsyndroom in Nederland. Daarbij zorgen de infecties, de autoimmuunziekten en de vormen van leukemie bij mensen met downsyndroom voor veel ziektelast en vroegtijdig overlijden. Het is dus zaak dat iedereen die met mensen met downsyndroom werkt kennis van zaken heeft over deze specifieke down-problematiek en deze tijdig kan herkennen en behandelen. Op deze manier kunnen wij als dokters onze bijdrage leveren aan de verbetering van de kwaliteit van leven voor mensen met downsyndroom.

AANBEVELINGEN VOOR TOEKOMSTIG ONDERZOEK

Wat is er nodig voor toekomstig onderzoek? We weten nog heel veel niet. Met name is het moeilijk te voorspellen bij welke kinderen met downsyndroom je problemen kunt verwachten door veranderingen in het afweersysteem. Welke kinderen binnen deze groep hebben een verhoogde kans op leukemie? Hoe wordt de kwaliteit van leven op de lange termijn (bijvoorbeeld spraak-taal, motorische en geestelijke ontwikkeling) bij deze groep beïnvloed door het optreden van infecties en autoimmuunziekten zoals schildklierproblemen en suikerziekte? En waar zit de fout in de samenwerking tussen de B- en de T-lymfocyten: meer bij de B-, meer bij de T-lymfocyt of bij beiden? En kunnen we door gerichte vaccinaties, het voorkomen van (luchtweg-) infecties, maar ook bijvoorbeeld door langdurige borstvoeding, de ongunstige veranderingen in het afweersysteem en de klinische gevolgen daarvan voorkomen?

Momenteel wordt door onze onderzoeksgroep verder onderzoek gedaan naar de ontwikkeling van B-lymfocyten, lange termijn consequenties van infecties op kwaliteit van leven en wordt geprobeerd duidelijker te krijgen hoe veel vaker kinderen met downsyndroom ziek zijn in vergelijking tot hun leeftijdsgenoten zonder downsyndroom. Ook is er een landelijk DownteamOnderzoeksConsortium (DOC) opgericht om gezamenlijk op te trekken om de kwaliteit van zorg voor mensen met downsyndroom te verbeteren door onderzoek en landelijke richtlijnen.

12

CURRICULUM VITAE
LIST OF PUBLICATIONS
LIST OF ABBREVIATIONS

CURRICULUM VITAE

Maaike Rekers-Kusters werd op 6 februari 1981 geboren in het Brabantse Rijkevoort. Maaike woont in Lent, en heeft een heerlijke tuin die ze deelt met haar man André Rekers, de *man achter de schermen* bij dit proefschrift! Haar favoriete film is Le fabuleux destin d'Amélie Poulain.

Maaike wist al op jonge leeftijd dat ze dokter wilde worden, hoewel plantkunde, design en architectuur haar ook trokken. Wat voor een dokter, dat was toen nog niet duidelijk.

De Aha-erlebnis kwam bij het co-assistentschap Kindergeneeskunde in het Twee-steden-ziekenhuis in Tilburg, met name door het aanstekelijke enthousiasme en de tomeloze toewijding van dokter Jaap van Lier.

De eerste stappen in de kindergeneeskunde werden gezet in het Maxima Medisch Centrum in Veldhoven en het Jeroen Bosch Ziekenhuis 's-Hertogenbosch als arts-assistent niet in opleiding (ANIOS). In 2010 kon worden gestart met de opleiding tot kinderarts (opleider T. Mulder) in het MUMC+ in Maastricht, de tweede helft van de opleiding wordt vervolgd in het Radboudumc (opleider J. Draaisma) in Nijmegen.

Het wetenschappelijk onderzoek werd verricht naast haar full-time klinische werk-zaamheden, met als uitvalsbasis het Jeroen Bosch Ziekenhuis onder de bezielende leiding van Dr. Esther de Vries.

Na haar opleiding tot kinderarts wil Maaike zich verder specialiseren binnen de immunologie, een tak van sport waar Maaike zeer gepassioneerd over is.

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LIST OF ABBREVIATIONS

Ad-REF	Reference values from healthy adults
ADHS	Antibody diluted human serum
AMC	Age-matched control
BD	Becton Dickinson
CD	Cluster of differentiation
CMV	cytomegalovirus
CSR	Class switch recombination
CVID	Common variable immunodeficiency disorders
DGS	DiGeorge syndrome
DS	Down syndrome
DSCAM	Down syndrome cell adhesion molecule
ELISA	Enzyme-linked immunosorbent assay
ENT	Ear-nose-throat
FACS	Fluorescence-activated cell sorting
FI	Fold Increase
FITC	Fluorescein isothiocyanate
GALT	Gut-associated lymphoid tissue
GMT	Geometric mean titer
HBsAg	Hepatitis B surface antigen
HI	Hemagglutination-inhibition
HIV	Human immunodeficiency virus
ICAM-1	Intercellular adhesion molecules-1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPD	Invasive pneumococcal disease
KREC	kappa-deleting recombination excision circles
LFA-1	Lymphocyte function-associated antigen-1
MAb	Monoclonal antibodies
MALT	Mucosa-associated lymphoid tissue
MenC	Meningococcal serogroup C conjugate vaccine
MFI	Mean fluorescence intensity
NK	Natural killer
OPA	Opsonophagocytosis assay
PCV-7	Heptavalent pneumococcal conjugate vaccine
PE	phycoerythrin
PE-Cy5	PE-cyanin 5
PHA	Phytohemagglutinin
PI	Phagocytosis index
PPV-23	23-valent pneumococcal polysaccharide vaccine
RPMI	Roswell Park Memorial Institute medium
RTE	Recent thymic emigrants
SHM	Somatic hypermutation
SPAD	Specific anti-polysaccharide antibody deficiency
Tc	Cytotoxic T
TD	T-cell dependent
Th	Helper-T
TI	T-cell independent
TNF	Tumour necrosis factor
TREC	T-cell-receptor excision circle
TT	Tetanus Toxoid