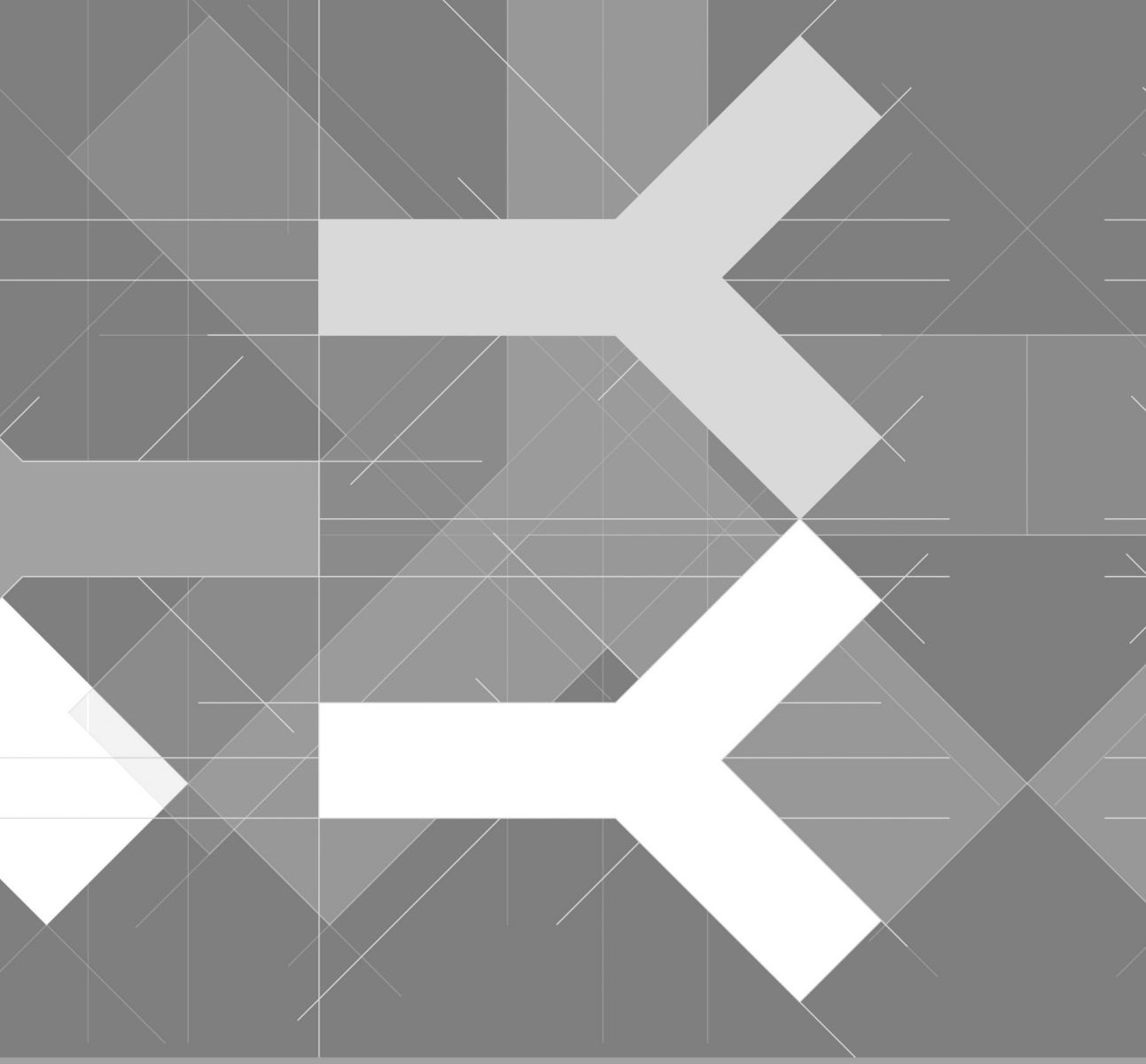

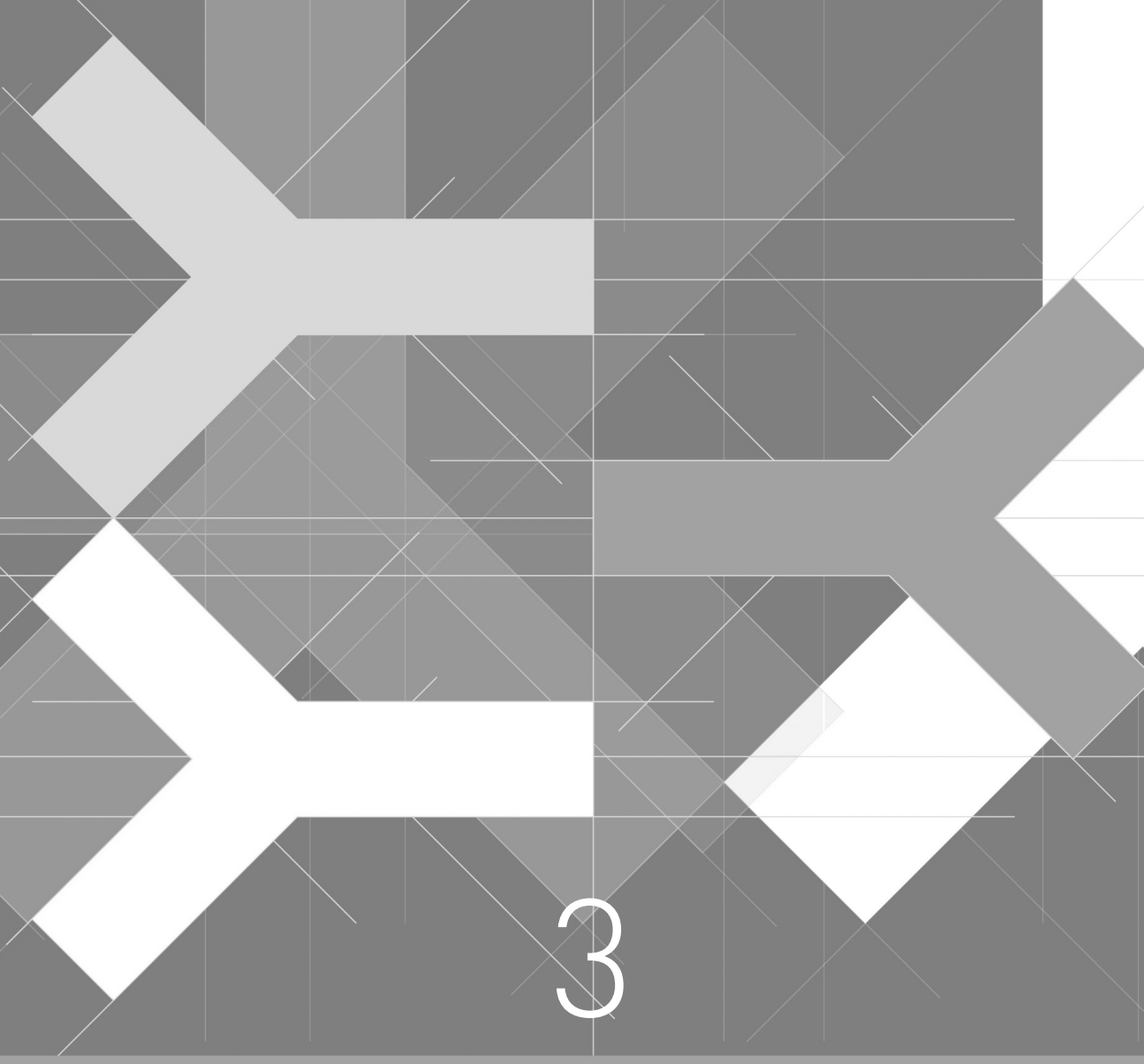
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SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

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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