Univerzita Karlova

2. lékařská fakulta

Studijní program: Imunologie

MUDr. Adam Klocperk

Adaptivní imunita u pacientů s primárními imunodeficiencemi

Adaptive immune system in patients with primary immunodeficiencies

Dizertační práce

Školitelka: Prof. MUDr. Anna Šedivá, DSc.

Konzultant: Doc. MUDr. Tomáš Kalina, PhD

Praha, 2019

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracoval samostatně a že jsem řádně uvedl a citoval všechny použité prameny a literaturu. Současně prohlašuji, že práce nebyla využita k získání jiného nebo stejného titulu.

Souhlasím s trvalým uložením elektronické verze mé práce v databázi systému meziuniverzitního projektu Theses.cz za účelem soustavné kontroly podobnosti kvalifikačních prací.

V Praze, 27.5.2019

MUDr. Adam Klocperk

KLOCPERK, Adam. *Adaptivní imunita u pacientů s primárními imunodeficiencemi. [Adaptive immune system in patients with primary immunodeficiencies].* Praha, 2019. 53 s., 3 příl. Dizertační práce (Ph.D.). Univerzita Karlova, 2. lékařská fakulta, Ústav imunologie. Školitelka Šedivá, Anna.

ABSTRACT (ENG)

This thesis summarizes the results of a project dedicated to adaptive immune system of patients with partial DiGeorge syndrome caused by deletion of 22q11.2.

The introduction sets the DiGeorge syndrome into a broader context of international pathophysio-clinical classification of primary immunodeficiencies and goes into detail describing its history, causes, clinical phenotype, therapeutic options and changes of the immune system.

The attached manuscripts illustrate the premature aging of the T cell population, but also impaired development of B cells with low class-switched memory and high naïve subpopulations, along with high serum levels of BAFF, a B cell survival factor. The surprising lack of T independent marginal zone-like (MZ-like) B cells is reflected in decreased natural anti-α-Gal antibodies. The faulty B cell maturation and imperfect germinal center response is not caused by a deficit of follicular helper T cells, which are in fact increased in DiGeorge syndrome patients, and in most cases doesn't lead to hypogammaglobulinaemia. Despite the high incidence of autoimmune disease, in particular thyroiditis and thrombocytopenia, and a trend towards hypergammaglobulinaemia in adolescence and adulthood, we saw normal proportion of regulatory T cells (Tregs) and normal expression of the transcription factor Helios, a marker of thymus-derived Tregs.

The outcome of this thesis project is an enrichment of our knowledge of the immune system dysregulation seen in patients with partial DiGeorge syndrome, the most common primary immunodeficiency with syndromic features, as obtained on its largest Czech cohort. The novel findings are correlated with clinical course of the disease and routinely available laboratory parameters, thus allowing for higher standard of care and monitoring for all patients.

Keywords: immunity, lymphocyte, digeorge, immunodeficiency, thymus

ABSTRAKT (CZ)

Tato dizertační práce shrnuje výsledky studií věnujících se adaptivnímu imunitnímu systému pacientů se syndromem DiGeorge způsobeným delecí 22q11.2.

V úvodu je syndrom DiGeorge zasazen do kontextu mezinárodní patofyziologicko-klinické klasifikace primárních imunodeficiencí, a ve větším detailu je rozebrána jeho historie, příčiny vzniku, klinický fenotyp, terapeutické možnosti a podrobně též změny v imunitním systému pacientů.

Přiložené studie dokazují předčasné stárnutí T lymfocytární populace, ale též nedokonalé vyzrávání B lymfocytů se sníženým počtem přesmyklých paměťových a naopak zvýšením naivních subpopulací a sérové hladiny jejich podpůrného B lymfocytárního cytokinu BAFF. Překvapivé snížení T independentních MZ-like B lymfocytů u pacientů dokládáme též signifikantním snížením přirozených anti-α-Gal protilátek. Porucha maturace B lymfocytů a nedokonalá reakce zárodečného centra není způsobena deficitem folikulárních pomocných T lymfocytů (Tfh), které jsou u pacientů proti kontrolám naopak zvýšeny, a ve většině případů nevede ke snížení sérových hladin IgG. Přes zvýšenou incidenci autoimunitních onemocnění a sklon k hypergamaglobulinemii mají však pacienti se syndromem DiGeorge normální zastoupení regulačních T lymfocyů (Tregů), jakož i expresi transkripčního faktor Helios, který je znakem thymických Tregů.

Výsledkem projektu je rozšíření znalostí o imunitním systému pacientů s parciálním syndromem DiGeorge, nejčastější primární imunodeficienci se syndromickými rysy, na jeho největší české kohortě. Nové poznatky jsou korelovány s klinickým průběhem a rutinně dostupnými laboratorními parametry, což umožňuje kvalitnější péči a monitoraci pro všechny pacienty.

Klíčová slova: imunita, lymfocyt, digeorge, imunodeficience, thymus

Adaptive immune system in patients with primary immunodeficiencies

TABLE OF CONTENTS

INTRODUCTION INTO STUDY OF PRIMARY IMMUNODEFICIENCIES

History of PIDs

Primary immunodeficiencies are a heterogeneous group of diseases caused by a missing or dysfunctional component of the human immune system. Even though we still consider them to be inextricably tied to increased susceptibility to bacterial, viral or fungal infections, it has become apparent in the past 20 years that in many primary immunodeficiencies there is also a very significant component of autoimmunity or immune dysregulation with a multitude of clinical manifestations, which may in some cases even overshadow the susceptibility to infections. Clearly, the definition of primary immunodeficiencies is a fluid and malleable one and can encompass a whole range of disorders of innate, adaptive, humoral or cellular immune systems.

Even though it was already the ancient Chinese who recognized the importance of protective immunity and practised a primitive form of vaccination by having children inhale dust made from smallpox skin lesions, in the western culture such refined recognition didn't come until Edward Jenner's famous observation of cowpox-exposed milkmaids being immune to smallpox in 1798(Downie 1951). Understanding of the mechanism with which the immune system operates didn't come until over one hundred years later, with Élie Metchnikoff receiving a Nobel prize in 1908 for his discovery of polymorphonuclear leukocyte phagocytosis and Paul Ehrlich joining him for his research on humoral immunity.

Although we already had some idea of how the human immune system operates and in what ways it reacts (or indeed does not react) to various stimuli, a systematic and complete description of particular immune diseases was missing. The first primary immunodeficiency that was well-described in the literature was what is now called X-linked agammaglobulinaemia, which was first documented by Ogden C. Bruton(Bruton 1952). In his seminal paper from 1952 in a journal called Pediatrics he descibed a case of a boy with missing gammaglobulins and recurrent infections, sepses, repeated positive hemocultures showing pneumococci and with no specific antibody response to typhoid vaccine. This patient responded very well to application of gammaglobulins, which seemed to protect him against further infections. The disease was aptly Bruton's agammaglobulinaemia, due to the patient's lack of gammaglobulins.

This type of agammaglobulinaemia accounts for roughly 85% of all agammaglobulinaemias, the most severe forms of primary antibody defects, in which all immunoglobulin isotypes are missing, as are mature B cells. Of course we are now aware of its genetic cause, which is the mutation in the eponymous Bruton's tyrosin kinase (*BTK*) gene, responsible for signal transduction downstream from

the B cell receptor(Vetrie et al. 1993). Due to the gene's location on the X chromosome the disease affects only males with gonosomal recessive mode of inheritance, while female carriers are unaffected due to positive selection of viable B cells in the bone marrow. In males, however, the pre-B cells are unable to transfer signal from the pre-B cell receptor through the SYK and LYN kinases onto the missing BTK protein and further downstream towards the phosphoinositide 3-kinase (PI3K) and phospholipase Cγ2 (PLC2) pathways, which results in block of progression into immature B cell stage. Interestingly, in some patients the mutation in *BTK* may have a milder phenotype and result in only partial defect of antibody response(López-Granados et al. 2005), such as in one reported patient who only lacked polysaccharide-specific antibodies(Wood et al. 2001). This genotype-phenotype variability is very common in many primary immunodeficiencies, rendering prognoses of clinical courses difficult even in patients with determined genetic sequence.

Classification of PIDs

In the 65 years since Ogden's first described primary immunodeficiency, our ever-increasing knowledge of the human immune system made possible especially by newly available technologies such as multicolor flow cytometry and especially since the start of the third millenium next generation sequencing. This lead to rapid discovery of hundreds of other primary immunodeficiences, as documented by the 354 distinct inborn errors of immunity listed in the 2017 Report on Inborn Errors of Immunity that is produced biannually by the IUIS, International Union of Immunological Societies(Picard et al. 2018). The list keeps growing at the speed of approximately 30 new entities per year and this growth is showing no signs of stopping.

The complexity and interconnectedness of the immune system makes classification into distinct groups often difficult, with many branches of the immune system frequently being affected by a single mutation. In the 1970, first attempt at summarizing the variety of primary immunodeficiencies has been made on behalf of the World Health Organization by Fudenberg et al., who divided 14 different primary immunodeficiencies based on the presence of T cell, B cell or stem cell defect(Fudenberg et al. 1970). In contrast, the most recent endeavour by the international immunology community now divides primary immunodeficiencies into eight categories (Table 1)(Picard et al. 2018).

Immunodeficiencies affecting cellular and humoral immunity

Immunodeficiencies affecting cellular and humoral immunity are also called combined immunodeficiencies and generally involve T cell dysfunction. The most important group of disorders in this category are severe combined immunodeficiencies (SCIDs), due to their high mortality rate if untreated. SCIDs can be further subdivided based on presence of T cells, B cells and NK cells. The most common form of SCID is the X-linked mutation in *IL2RG* gene coding the common gamma subunit of

several cytokine receptors involved in development of T cells. Patients with *IL2RG* mutations are said to have T-B+NK- SCID, as have no or very few T cells and NK cells but present B cells. The condition is life-threatening and requires urgent hematopoietic stem-cell transplantation, or, rarely, gene therapy.

Combined immunodeficiencies with associated or syndromic features

Combined immunodeficiencies with associated or syndromic features have added non-immunologic phenotypes, such as facial dysmorphia, musculo-skeletal abnormalities, affection of non-immunogenic cellular lineages and others. This group also includes DiGeorge syndrome which will be described in greater detail below.

Predominantly antibody deficiencies

Predominantly antibody deficiencies are the most prevalent disorders, featuring selective IgA deficiency, the most common asymptomatic immunodeficiency (with prevalence of cca 1:500), and common variable immunodeficiency (CVID), the most common symptomatic immunodeficiency. CVID is a genetically hugely heterogeneous group of disorders characterized by impaired specific antibody response, low serum immunoglobulins and increased susceptibility to infections, usually respiratory, which however are usually not life-threatening. Most patients are only diagnosed as adults and in most of them, immunoglobulin substitution is sufficient to avoid morbidity. In about one third, however, immune dysregulation, lymphoproliferation and cancers are present, worsening the overall prognosis significantly(Cunningham-Rundles 2012). It is expected, that as our understanding of the immune system grows, more and more immune gene mutations will be described in patients with CVID and the group will continue to splinter.

Diseases of immune dysregulation

Diseases of immune dysregulation are a fascinating group of disorders where increased susceptibility to infections is only one part of a greater clinical picture which frequently features autoimmunity or lymphoproliferation as signs of underlying dysregulation of the immune system. One such disease is autoimmune polyendocrinopathy candidiasis and ectodermal dysplasia (APECED) as caused by mutations in the *AIRE* gene leading to breakdown of central immune tolerance. Patients with APECED develop organ-specific autoimmunities leading to hypoparathyreoidism, adrenal gland insufficiency or type 1 diabetes(Aaltonen et al. 1997). The immune deficiency and chronic mucocutaneous candidiasis is caused by autoantibodies against IL-17 which break down the Th17 response against fungal pathogens. Unfortunately, treatment is generally limited to substitution of lost endocrine function, but may involve immunosuppression in an attempt to stop further deterioration.

Congenital defects of phagocyte number or function

Congenital defects of phagocyte number or function include numerous primarily hematological disorders causing primary neutropenias such as Kostmann's disease, X-linked neutropenia and others, but also defects affecting the proper function of neutrophils. A hallmark disorder in this category is chronic granulomatous disease (CGD), the most common form being caused by an X-linked mutation in cytochrome b-245 beta chain (*CYBB*) resulting in impaired respiratory burst and thus compromised destruction of phagocytosed bacteria(Goldblatt and Thrasher 2000). Neutrophils struggling to remove pathogens form multinucleated conglomerates, which together create localized lesions, granulomas. Most patients are treated with prophylactic antibiotic therapy and in more severe cases with hematopoietic stem cell transplantation(Thrasher and Williams 2017), however substantial progress has been made in the past several years with gene therapy for carriers of the *CYBB* mutations.

Defects in intrinsic and innate immunity

Defects in intrinsic and innate immunity are quite interesting in that even though they affect the innate immune system which is a broad spectrum unspecific shield against all pathogens, several of the diseases in this category actually manifest with very specific and narrow profiles of infectious pathogens. For example, toll-like receptor 3 (TLR3) deficiency results in poor sensing of double-stranded RNA and can present as severe Herpes simplex virus 1 encephalitis(Casanova 2015). In a fascinating twist, while the loss of function mutations in signal transducer and activator of transcription 1 (STAT1) dampen interferon γ signalling in macrophages and disable their response to intracellular pathogens such as mycobacteria, gain of function mutations in the same gene result in overactive STAT1 signaling, which in turn supresses Th17 response vital for defense against fungal pathogens.

Autoinflammatory disorders

Autoinflammatory disorders stride the border of primary immunodeficiency definition, because in majority of them increased susceptibility to infections is either not present at all, secondary to vascular and cellular damage from pervasive inflammation or consumption, or it is simply by far not the dominant symptom. The first autoinflammatory disorder with a defined genetic cause was the Familiar Mediterranean Fever (FMF) caused by mutation of *MEFV* gene encoding the protein pyrin. Pyrin is a molecule capable of sensing disturbances in celular homeostasis (such as those induced by an ongoing infection) through the activity of RhoA GTPase, to which it responds with promoting inflammasome assembly and cleavage of pro-IL-1 into its active IL-1β form. Thus, the outcome of mutated pyrin is reckless production of proinflammatory cytokines, fever and systemic inflammation(Manthiram et al. 2017). Thankfully, recent endeavours of several pharmacological companies brought us medication capable of blocking the activity of IL-1β, such as the IL-1β receptor antagonist anakinra, or

canakinumab, a recombinant anti-IL-1β antibody. These compounds can suppress the inflammation and thus, among other effects, prevent deposition of inflammation-induced proteins into tissues – amyloidosis, a severe complication of all autoinflammatory diseases.

Complement deficiencies

Finally, complement deficiencies are frequently not well known among practising clinical immunologists due to the complexity of the complement cascade and variable manifestations overlapping with other medical specialities. Regardless, most complement deficiencies present with recurrent or disseminated infections with encapsulated bacteria (in particular *Neisseria* species) and impaired immune complex and apoptotic body removal capabilities, resulting in self-antigen recognition and development of autoimmune diseases such as systemic lupus erythematosus (SLE). The penetrance is not 100% however and for example while the populational prevalence of C2 deficiency is upwards of 1 in 10,000, the rate of diagnosis is much lower(Alper et al. 2003; Göran et al. 2005).

Complement deficiencies also include the deficiency of C1 inhibitor (caused by mutation of the *SERPING1* gene), which presents as hereditary angioedema, or mutations of C3, thrombomodulin or CD46, which present as atypic hemolytic-uremic syndrome. Neither of these present primarily with increased susceptibility to infections, but rather unchecked activation of the complement cascade resulting in swelling or destruction of glomerular microvasculature.

Group	**# of entities**	**Examples**
Immunodeficiencies affecting cellular and humoral immunity	49	SCIDs (*IL2RG*, *JAK3*, *RAG1* etc.), *ZAP70* def., hyper-IgM sy., *DOCK8* def.
Combined immunodeficiencies with associated or syndromic features	67	DiGeorge sy., WAS sy., ataxia teleangiectasia, Nijmegen sy., *STAT3* def., *NEMO* def.
Predominantly antibody deficiencies	40	XLA, CVID, APDS, IgG subclass def.
Diseases of immune dysregulation	40	FHL, APECED, ALPS, *XIAP* def.
Congenital defects of phagocyte number or function	39	CGD, LAD, *GATA2* def.
Defects in intrinsic and innate immunity	52	MSMD (*IL12RB*, *IFNGR1*, *STAT1* etc.), WHIM sy., *TLR3* def., *STAT1* GOF
Autoinflammatory disorders	37	SAVI, FMF, hyper IgD sy., cryopyrinopathies, ADA2 def.

Complement deficiencies	30	C2 def.

Table 1: Classification of primary immunodeficiencies, based on (Picard et al. 2018). SCID = severe common immunodeficiency, def. = deficiency, sy. = syndrome, WAS = Wiskott-Aldrich syndrome, XLA = X-linked agammaglobulinaemia, CVID = common variable immunodeficiency, APDS = activated PI3Kδ syndrome, FHL = familiar hemophagocytic lymphohistiocytosis, APECED = autoimmune polyendocrinopathy with candidiasis and ectodermal dysplasia, ALPS = autoimmune lymphoproliferative syndrome, CGD = chronic granulomatous disease, LAD = leukocyte adhesion deficiency, MSMD = mendelian susceptibility to mycobacterial disease, WHIM = warts, hypogammaglobulinemia, infections, myelokathexis syndrome, GOF = gain of function, SAVI = STING-associated vasculopathy with onset in infancy, FMF = familial mediterranean fever

DIGEORGE SYNDROME

History

When in 1965 Cooper et al. published a study proposing that two different sets of lymphocytes exist in mammals, those coming from thymus and responsible for cellular immunity and those coming from tonsils responsible for humoral immunity, Angelo DiGeorge commented with personal observation of 4 infants with missing parathyroid glands, in whom autopsies and early roentgenograms showed also the absence of thymus(A. DiGeorge 1965). In these patients, DiGeorge documented normal serum immunoglobulin levels and present lymphocytes, but absent cellular immune response manifesting as recurrent fungal infections with the *Monilinia* fungus, yet no skin reaction to administration of the *Monilia* antigen or signs of delayed type hypersensitive reaction to 1-chloro-2,4-dinitrobenzene, a powerful inducer of type IV hypersensitivity that was historically used for treatment of warts and assessment of T cell function. Since then, the term “DiGeorge syndrome” has been coined for the congenital failure of third and fourth pharyngeal pouch development leading to athymia and immunodeficiency. DiGeorge summarized his observations regarding development of lymphocytes in his patients in another paper from 1967(Lischner, Punnett, and DiGeorge 1967), where he postulated at the time popular hypothesis that a soluble “thymic factor” is produced by the thymus and is able to support development of lymphocytes *in vitro*. As an addition, “transfer factor” which constitutes a lysate from lymphocytes reactive to a particular antigen, was also supposed to induce reactiveness to that antigen in previously incompetent unreactive lymphocytes. Despite extensive attempts at mixed *in vitro* cultures making use of biologic material from athymic infants, however, DiGeorge and his team found no evidence for such a factor and concluded that “immunologically competent lymphocytes have their primary origin in the thymus or at least require circulation through the intact thymus”. Thus,

the importance of thymic stroma for the development of lymphocytes and adaptive immune response was first proposed.

Causes

The underlying cause of DiGeorge syndrome, or congenital absence of thymus coupled with missing parathyroid glands, remained unknown for over 35 years since its discovery, even though DiGeorge et al. already recognized that these two structures share a common developmental origin, the third pharyngeal pouch. Pharyngeal pouches are protrusions of the primordial endoderm, which in humans form between the pharyngeal arches at approximately 26 days after gestation. From the third and fourth pouches, the thymus, parathyroid glands and great vessels of the heart eventually develop, which explains why these structures are jointly affected in DiGeorge syndrome patients. Whereas the mesoderm and neural crest cells give rise to eventual connective tissue and vascular endothelium, the thymic epithelial stroma capable of supporting thymocytes develops from the endoderm under directive of the transcription factor FoxN1. Even though FoxN1 is not required for establishment of the thymic epithelial cell (TEC) lineage(Blackburn et al. 2002), or indeed the third pharyngeal pouch or thymic primordium itself(Cordier and Haumont 1980), its absence (such as in the *nu/nu* nude mice) blocks the development of rudimentary TECs into fully functional thymic microenvironment. Human *FOXN1* mutations reflect this role of FoxN1 by resulting in severe combined immunodeficiency with absence of thymus, with the additional feature of absence of hair and nail dystrophy, caused by the role FoxN1 plays in proper function of hair follicles(Pignata, Fiore, et al. 1996).

Pinning down the exact cause, prevalence, clinical and immunological phenotype of DiGeorge syndrome is complicated by its significant overlap with several other syndromes, which are sometimes used ambiguously in the literature and clinical practise. The clinical presentation of DiGeorge syndrome overlaps with velo-cardio-facial syndrome (VCFS), sometimes called Shprintzen syndrome, diagnosed clinically based on cleft palate, congenital heart defects and facial dysmorphy, and the conotruncal anomaly face syndrome sometimes called Takao syndrome, in which the cardiac defect is the focus of the syndrome's description. All of these syndromes have similar clinical presentation and are associated with hemizygous deletions in the 22q11.2 chromosomal region and can happen in a single family. Attempts have been made at unifying the nomenclature with neutral labels such as 22q11.2 deletion syndrome, or CATCH22 (cardiac defect, abnormal facies, thymic hypoplasia, cleft palate, hypocalcaemia)(Burn 1999), however no consensus has been reached, as documented by some papers by Shprintzen, who argues that all other syndromes are merely variations and re-labels of VCFS(Shprintzen 2008). For the sake of this thesis and its attached papers, patients with DiGeorge syndrome fulfil the European Society for Immunodeficiencies (ESID) diagnostic criteria for DiGeorge

syndrome, which list reduced number of CD3+ lymphocytes (T cells) <1500 cells/mm^3 as the only prerequisite, *conditio sine qua non* condition, whereas genetic defect (deletion in the 22q11.2 region), cardiac defect and hypocalcaemia of >3 weeks that requires therapy are only supporting criteria, not all of which need to be fulfilled for the diagnosis. These 4 findings reflect the pathophysiological basis of DiGeorge syndrome and are the best representation of the most robust complications.

As seen above, identified genetic cause is not necessary for the diagnosis of DiGeorge syndrome, however between 30 and 90% of patients with the diagnosis of DiGeorge syndrome do carry the aforementioned interstitial 22q11.2 deletion(D. A. Driscoll et al. 1993; D. a Driscoll, Budarf, and Emanuel 1992). This deletion is fairly common in the general population with incidence of around 1:4000(Tézenas Du Montcel et al. 1996) and in a large study published in 1997 summarizing the clinical phenotype of 558 patients with 22q11.2 deletions, only around 1% had phenotype suggestive of complete DiGeorge syndrome with major immunologic abnormalities(Ryan et al. 1997). A larger proportion, around 30% of patients, had a milder immunologic phenotype(Jyonouchi et al. 2009) with lower but present T cells, for which the term partial DiGeorge syndrome was coined early on(Lischner and DiGeorge 1969).

Most 22q11.2 deletions share a similar size due to regions of low copy number repeats. Around 90% of patients with VCFS have a 3 Mb deletion in the 22q11.2 region(Carlson et al. 1997), whereas most other patients have a smaller, proximally nested 1,5 Mb deletion(Meechan et al. 2007). Rarely, distal nested mutations or even mutations adjacent to the typical 3 Mb deletion location can be detected in patients with congenital conotruncal heart defects, however their phenotype is not always typical for classical DiGeorge syndrome(Rauch et al. 2005). Another rare cause of DiGeorge syndrome are deletions on the 10^{th} chromosome, which have a slightly different clinical phenotype and have been called DiGeorge syndrome 2(Pignata, D'Agostino, et al. 1996), however those have incidence of around 1:200000 and thus are at least 50 times more rare than the classical 22q11.2 deletion. It should also be noted that some patients with CHARGE (coloboma, heart defect, atresia choanae, retarded growth and development, genital hypoplasia, ear abnormalities/deafness) syndrome caused by mutations in the *CHD7* gene also overlap clinically with DiGeorge syndrome patients(Jongmans et al. 2006).

Identifying the critical region or a singular gene responsible for the DiGeorge syndrome hasn't yet proven fruitful. A localized deletion of the *TBX1*, a T-box gene which locates to 22q11 and is responsible for regulating expression of a variety of other genes, can result in the DiGeorge phenotype(Yagi et al. 2003), suggesting a unique role for *TBX1* in development of DiGeorge syndrome, however, since some of the distal mutations don't include *TBX1* yet present with DiGeorge phenotype, it is clear TBX1 is not the only culprit.

Finally, several non-genetic causes of DiGeorge syndrome have been identified. These include prenatal exposure to increased levels of retinoic acid(Coberly, Lammer, and Alashari 1996), which has been shown to downregulate Tbx1 in birds(Roberts et al. 2005), foetal alcoholic syndrome(Ammann et al. 1982) and maternal diabetes(Digilio et al. 1995), even though these are mostly individual case reports rather than larger population studies, so true pathophysiological connection remains uncertain.

Clinical phenotype

As described above, there is great variability of clinical phenotypes associated with deletions in 22q11.2, even when filtered down to patients with the more specific DiGeorge syndrome. The most common cause leading to genetic testing of 22q11.2 in newborns and infants are congenital heart defects, in particular the tetralogy of Fallot (aorta incumbens, pulmonary valve stenosis, ventricular septal defect, right ventricle hypertrophy), or hypocalcaemia.

The hypocalcaemia is incurred by hypoparathyroidism and presents in over half of 22q11.2del patients(Cheung et al. 2014). This may present as tetany and seizures, or more insidiously as difficulty in feeding and weakness. In some patients, the hypoparathyroidism may be latent until times of greater calcium requirement, such as after surgery, injury, or during adolescence or pregnancy. The severity usually decreases with age, however, and is rarely dangerous in later years. Heart defects, on the other hand, can be life-threatening and are the largest cause of syndrome-associated mortality in both children and adults(Repetto et al. 2009).

A secondary tell-tale sign of DiGeorge syndrome which can reinforce the suspicion and lead to diagnostic testing is the characteristic facies with low-set, round ears, telecanthus, short palpebral fissures, slanting eyes with hooded eyelids (both upward and downward slanting eyes have been described) and small mouth(Óskarsdóttir et al. 2008). Asymmetrical crying face due to depressor anguli oris muscle hypoplasia is seen in upwards of 20% 22q11.2del neonates(Giannotti et al. 1994).

Of course, once clinical suspicion of DiGeorge syndrome is aroused, the genetic studies will be promptly accompanied by testing of circulating T lymphocytes and potentially T cell receptor excision circles (TRECs) for fear of immunodeficiency and cementing of diagnosis. Particular immunologic findings will be discussed in the next chapter, however as already postulated, there is a vast spectrum of severity seen in DiGeorge syndrome immunodeficiency ranging from severe combined immunodeficiency with absent T lymphocytes, through Omenn-like syndrome with oligoclonal expansion of a small population of T cells and erythematous rash, maternal T cell engraftment all the way to mostly mild immunodeficiency, which is by far most common. Recurrent sinusitis and recurrent otitis are seen in roughly 25% of partial DiGeorge syndrome patients over 9 years of age, recurrent

bronchitis in 7% and recurrent pneumonias in 4%(Jawad et al. 2001). Infections with opportunistic pathogens are a threat in those patients with particularly low T cells and present a significant source of morbidity and mortality(Eberle et al. 2009).

When immediate concerns such as disturbed biochemistry, cardiac anomaly or severe immunodeficiency are alleviated, other features may become apparent. Chief among those is developmental delay seen already in toddlers and encompassing both motor and language delays(Solot et al. 2000). Surgical correction of velopharyngeal cleft or velopharyngeal insufficiency may be of benefit, although even later in life these patients show reduced academic performance, particularly in mathematics and skills requiring abstract reasoning(De Smedt et al. 2003). The more severe end of psychological issues seen in DiGeorge/VCFS patients ranges into psychotic disorders, with stark increase in prevalence of schizophrenia in patients compared to general population(Murphy, Jones, and Owen 1999).

Autoimmune disorders and presence of autoantibodies are more common in DiGeorge/22q11.2del syndrome patients(Gennery et al. 2002). In particular, studies have reported increased prevalence of juvenile idiopathic arthritis, which associated with low IgA(K. Davies et al. 2001; Kathleen E. Sullivan et al. 1997; Verloes et al. 1998), idiopathic thrombocytopenic purpura (ITP)(Jawad et al. 2001) and to lesser extent autoimmune hemolytic anemia(Jawad et al. 2001) and hypothyroidism or hyperthyroidism suggestive of Graves disease(Kawame et al. 2001). The cause of autoimmune disease in DiGeorge syndrome is not clearly elucidated. Some mechanisms will be discussed in the following section.

Atopic disease is also more common in patients with DiGeorge syndrome, taking on the form of atopic dermatitis or asthma, but less so allergic rhinitis(Staple et al. 2005). Production of Th2 cytokine IL-4 is higher in adults but not infants with 22q11.2 deletion syndrome(Zemble et al. 2010). Another study in which the investigators extracted data from electronic health records found evidence of atopic disease in two thirds of 186 22q11.2del syndrome patients, of whom 40% had asthma, which is much higher than the 8.5% prevalence of asthma in compared general population(Morsheimer et al. 2017).

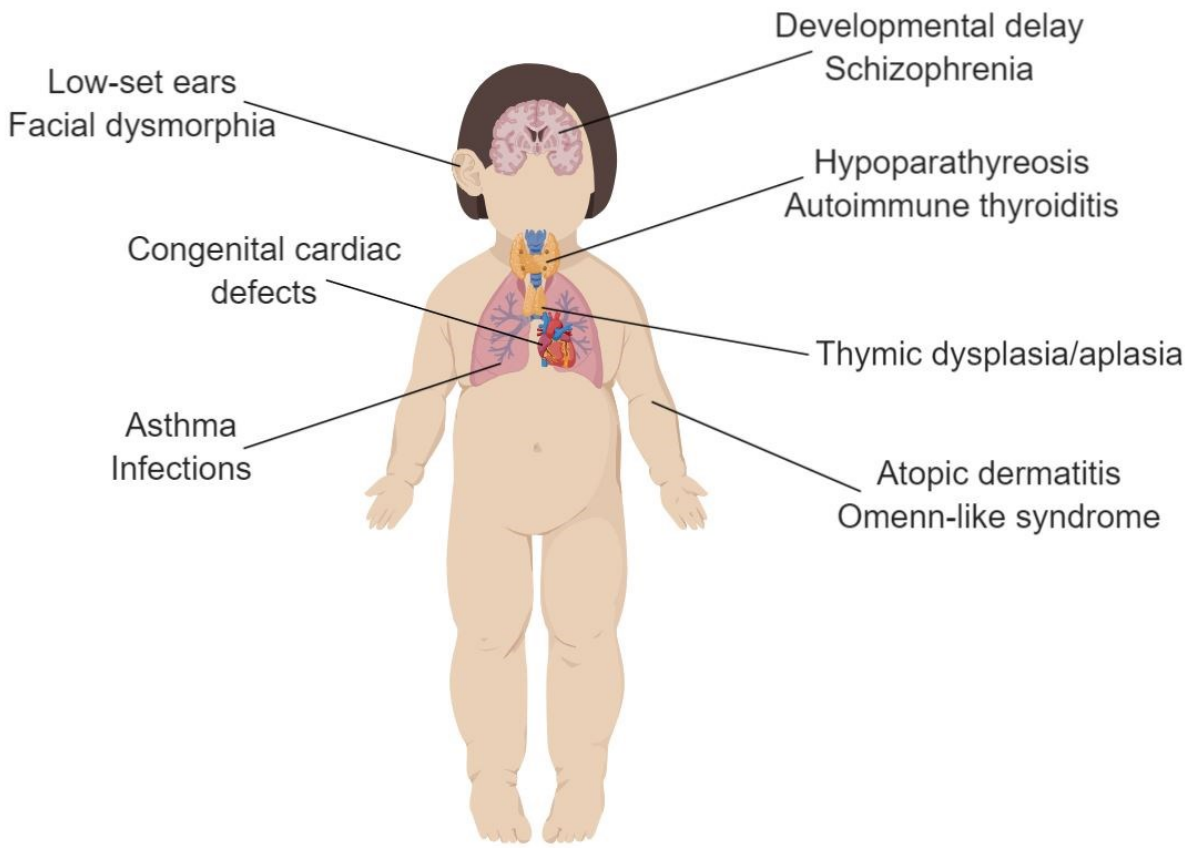

Figure 1: Clinical phenotype of patients with DiGeorge syndrome. Patients are frequently affected by developmental delay and have increase chance of developing schizophrenia. Congenital hypoparathyreosis due to dysplasia of parathyroid glands and autoimmune thyroiditis characterize endocrine complications seen in DiGeorge syndrome. Congenital heart defects are caused by erroneous development of the third pharyngeal pouch during embryogenic period. Thymic dysplasia or aplasia lead to immunodeficiency and immune dysregulation that can manifest as increased susceptibility to infections, asthma, atopic dermatitis or Omenn-like syndrome. Low-set ears and other cranio-facial dysmorphy may serve as diagnostic clues.

Immunity

Decreased thymic output, T cells

The primary change of the immune system in DiGeorge syndrome is the decrease of thymic output, caused by thymic aplasia or dysplasia, which results in low numbers of T cells in the peripheral blood. The lymphopenia and defect of cellular immunity was one of the hallmarks described already by Angelo DiGeorge in the 60's(A. M. DiGeorge et al. 1967). We have since recognized that the size and function of thymi in 22q11.2del patients are highly variable, ranging from normal thymi with preserved architecture, through smaller, ectopic thymi with normal architecture, thymi with disturbed microscopic architecture (the cortico-medullary division, Hassal corpuscules and other) all the way to complete absence of thymi. This in turn affects the level of T cell lymphopenia and is the reason why some 22q11.2del patients have normal T cells and thus don't fulfil DiGeorge syndrome criteria and should rather be classified as VCFS or Takao syndrome, whereas others suffer from clinical picture of severe combined immunodeficiency. For the remainder of this chapter, unless specifically stated otherwise, DiGeorge syndrome refers to its less severe, partial form, which shows some preserved T cell production. Complete DiGeorge syndrome generally takes the form of SCID with no detectable T cells, or as mentioned above Omenn-like syndrome with oligoclonal expansion of host or maternally engrafted T cells.

Thymus

The mechanism through which T cells develop from pro-thymocytes into mature naïve T cells has been thoroughly explored and reviewed numerous times(Germain 2002; Klein et al. 2014; Ladi et al. 2006). T cell precursors are continuously released from the bone marrow and home into the thymus after the gradient of several chemokines which include CXCL12, CCL21 and CCL25. These guide the precursors into post-capillary venules located at the cortico-medullary junction, from which the cells enter thymus proper.

Thymus consists of densely packed developing thymocytes meshed between thymic epithelial cells (called cTEC and mTEC for cortical and medullary thymic epithelial cells respectively) encapsulated in connective tissue which divides the thymus into numerous smaller lobules. The stroma is further enriched by B cells, dendritic cells and macrophages, which can be found predominantly in the lighter-coloured medulla.

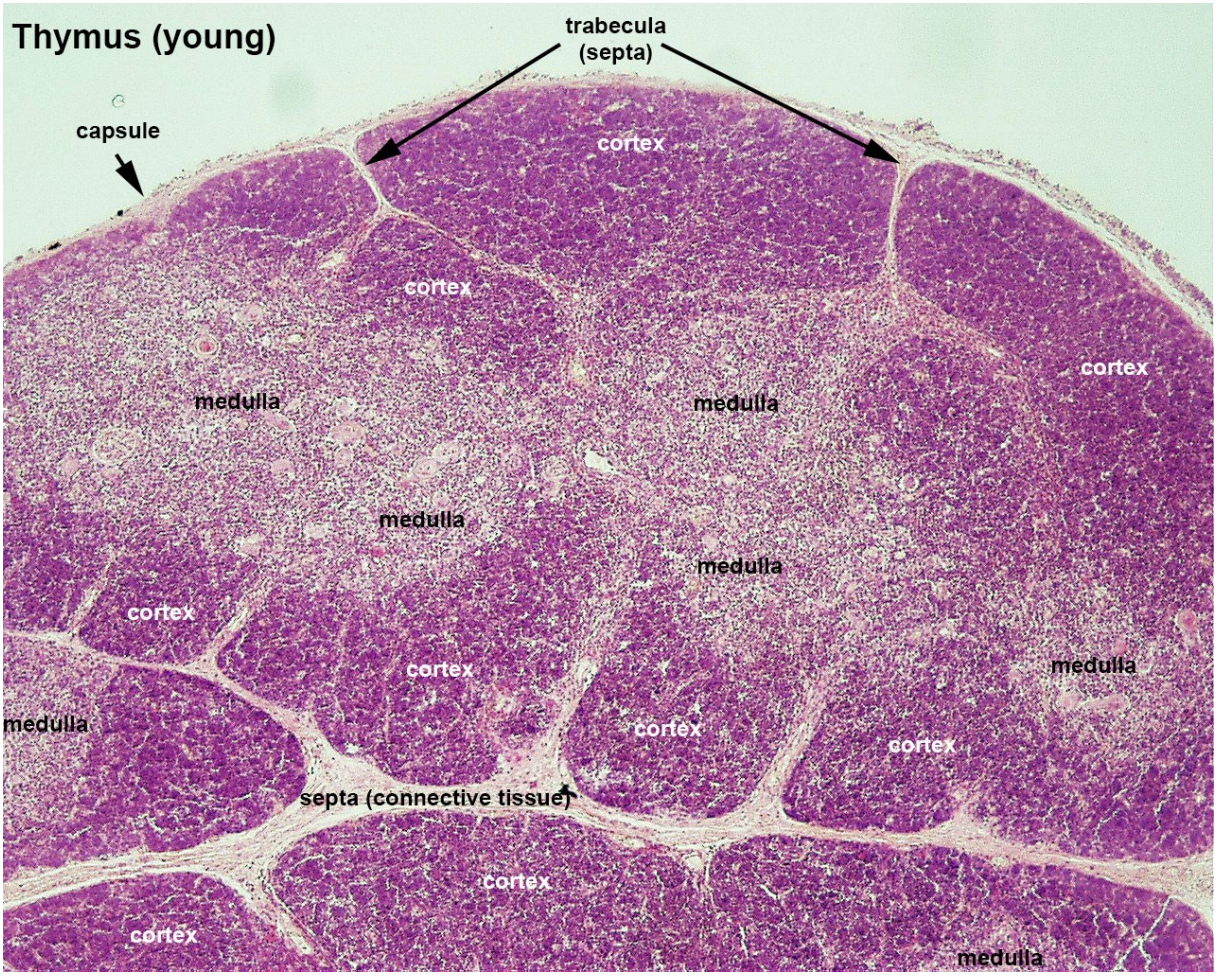

Figure 2: Histology of the thymus. The structure of thymus in young humans shows differentiation into lightly staining medulla and more densely packed darker cortex. The thymus is divided into larger lobules with septa consisting of connective tissue and wrapped in a connective tissue capsule. Originally published by Hill et al.(Hill 2019)

Thymic cortex, positive selection

The thymocytes that enter at the cortico-medullary junction express CD3 but neither CD4 nor CD8 and are thus called “double negative” (DN). Similarly to developing B cells, DN thymocytes rearrange their VDJ genes in a process called VDJ recombination, which facilitates the polyclonality necessary in T and B cells. Beta chain of the T cell receptor complex is rearranged first and expressed at the cell surface together with a constant pre-Tα chain. Should this rearrangement and pairing be successful, the pre-T cell receptor (pre-TCR) associates with CD3, is stimulated through oligomerization(Yamasaki et al. 2006) and sends positive survival signals into the nucleus which also trigger the expression of CD4 and CD8 molecules. This marks the transition of the thymocyte into “double positive” (DP) state.

DP thymocytes come into contact with cTECs, which on their surface express a wide variety of peptides presented in the context of both MHC-I and MHC-II molecules – the collective summary of peptide-MHC molecules is sometimes called “thymic peptide-MHC ligandome”. Not only do these cTECs have a substantial self-peptide-MHC-I presentation due to proteasomes containing a special thymus-restricted β5t subunit, but they also actively autophage self-molecules and present them in in context of MHC-II in a process called “macroautophagy”. Even though this allows for a particularly broad ligandome which ensures that many developing DP thymocytes can recognize a peptide-MHC complex, about 90% of DP thymocytes don’t pass positive selection checkpoint and die with apoptosis by neglect. The high turnover is enabled by thymic macrophages which digest the apoptotic bodies created by suicidal thymocytes unable to recognize a peptide. Those DP thymocytes that pass positive selection diverge into either CD4 or CD8 lineage based on whether their TCR recognizes peptides presented in complex with MHC-II or MHC-I respectively, becoming “single positive” (SP).

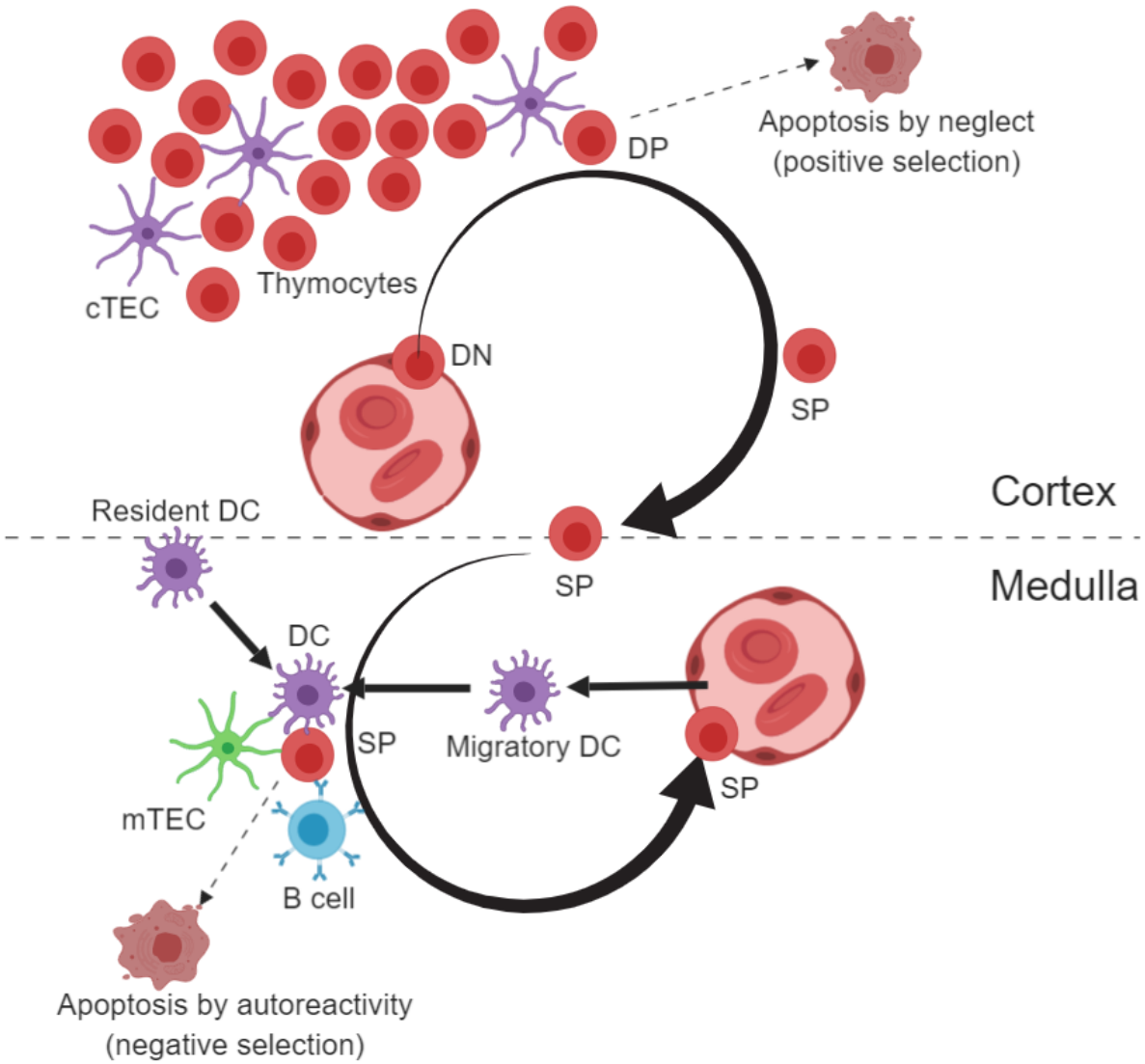

Figure 3: T cell development in the thymus. Prothymocytes enter from the bloodstream and journey through the cortex, where they encounter cTECs. If they can recognize the peptides presented in the context of MHC molecules, they pass the checkpoint of positive selection and progress into the medulla. Here they are exposed to mTECs, DCs and B cells presenting further MHC-antigen complexes which facilitate negative selection. Should that checkpoint also be surpassed, mature naïve T cells exit into the blood stream in search of their cognate antigen. Adapted from Klein et al(Klein et al. 2014). cTEC = cortical thymic epithelial cell. DN = double negative. DP = double positive. SP = single positive. DC = dendritic cell. mTEC = medullary thymic epithelial cell.

Thymic medulla, negative selection

SP thymocytes migrate after the CCR7 gradient into thymic medulla which hosts mTECs and a wide variety of other professional antigen presenting cells, such as B cells, thymic resident dendritic cells (DCs) or migratory DCs. The medulla is where the crucial central tolerance is established, selecting out and inducing apoptosis in T cells with autoreactive potential. The key players for this process are the antigen presenting cells, spearheaded by the mTECs. mTECs express high levels of Autoimmune regulator (*AIRE* gene coding Aire protein), which enables them to freely transcribe genes coding virtually all peripheral tissue antigens, yet maintain their own identity. Aire achieves this promiscuous gene express not through specific binding to DNA motifs and acting as a transcription factor, but rather by enabling RNA polymerase II already bound to DNA, but stalled at the promotor regions of tissue specific genes, to continue its activity(Giraud et al. 2011). Aire also partners with numerous other proteins, some of which – such as DNA topoisomerase IIα – can induce double-strand DNA breaks, relaxing the chromatin and allowing for yet easier transcription(Abramson and Goldfarb 2016).

These tissue specific antigens are digested and presented in the context of MHC molecules to the developing thymocytes. Interestingly, thymic resident dendritic cells can obtain these tissue specific antigens from mTECs under the control of Aire and present them to developing thymocytes themselves(Huber et al. 2011). B cells and migrating dendric cells circulate between the peripheral blood and thymic medulla, presenting antigens obtained directly *in situ* in the periphery.

When the SP T cells come into contact with these presented antigens, either on the surface of mTECs, B cells or resident or migratory dendritic cells, those T cells reacting with high affinity are “negatively selected” and undergo apoptosis. Those with medium affinity usually become T regulatory cells (Tregs), while those reacting with low affinity are destined to become canonical effector T cells.

Should they survive, they are finally released to emigrate from the thymus following the signals provided to them by sphingosine 1-phosphate type 1 receptor ($S1P_1$). S1P is plentiful in the blood and lymph, but maintained at low levels in the thymus and lymph nodes, thus facilitating egress from the lymphatic organs into circulation. At this stage, the T cells are called recent thymic emigrants (RTE) and can be distinguished in the peripheral circulation by their expression of CD31 (Platelet endothelial cell adhesion molecule (PECAM-1)) and CD45RA. CD31+ CD45RA+ CD4 T cells have longer telomeres and higher concentration of TRECs than their CD31- counterparts(Kohler and Thiel 2009). A similarly suitable marker is not yet known for CD8 T cells(Junge et al. 2007).

In summary, the thymus has been thoroughly demonstrated to be a vital organ for mature T cell development, crucial for selection of T cells able to recognize peptides and thus fight off infection or cancer, but at the same time tolerate the host tissues and not cause autoimmune disease.

T cell maturation in DiGeorge syndrome

We and others have shown that the low thymic output caused by small volume or dysplastic architecture which results in overall T cell lymphopenia is most notable in infancy, when DiGeorge syndrome infants have roughly one half of T cells seen in their healthy age-matched counterparts(Klocperk et al. 2014; Piliero et al. 2004). The overall levels of TRECs and RTEs, as primary markers of thymic output, are decreased in DiGeorge syndrome patients(Eberle et al. 2009; Lima et al. 2010; Piliero et al. 2004). The reduction of overall T cells is apparently driven already *in utero*, as documented by comparative decrease of T cells in DiGeorge syndrome patients compared to otherwise healthy newborns who underwent corrective cardiac surgery with thymectomy shortly after birth(K E Sullivan et al. 1999). In those children, post-natal thymectomy resulted in lower T cells than in healthy population, but still significantly higher than in DiGeorge syndrome patients.

This difference slowly wanes over the first 20 years of life or so. The current opinion is that this gradual "normalization" is in one part due to lowering of healthy average T cell counts with age, but also due to homeostatic proliferation of mature T cells especially in DiGeorge patients. This process of homeostatic proliferation results in dilution of TRECs and shortening of telomeres, both of which have been shown in DiGeorge syndrome compared to healthy (Piliero et al. 2004). This proliferation is at least in part sustained by heightened concentration of IL-7, a canonical T cell survival cytokine sensed by CD127 (IL-7 receptor) particularly highly expressed on naïve and long-lived memory T cells(Bachmann et al. 2014). IL-7 is elevated in DiGeorge syndrome patients with low T cells(Tantibhaedhyangkul et al. 2009) compared to those with normal T cell numbers. The proportion of memory CD45RA- or CD45RO+ T cells is high in all age groups of DiGeorge syndrome patients, as shown repeatedly by both our group and others(Klocperk et al. 2018).

The dysfunctional thymic microenvironment not only causes low numbers of T cells, but also impairs the process of generating a wide variety of T cell clones. Indeed patients with DiGeorge syndrome had significantly different usage of the various TCR Vβ families compared to healthy controls(A. McLean-Tooke et al. 2011), and had more oligoclonal and drop-out Vβ families(Zemble et al. 2010).

Functionality of T cells seems mostly preserved in partial DiGeorge syndrome. Proliferative response to mitogens is roughly normal(Chinen et al. 2003; K E Sullivan et al. 1999; Zemble et al. 2010). Some groups described aberrant cytokine production with skew towards IFNγ producing Th1 T cells in infancy but IL-4 producing Th2 T cells in adulthood(Zemble et al. 2010), however others did not(Kanaya et al. 2006).

The wide range of T cell defects seen in DiGeorge reflects the spectrum of clinical manifestations seen in individual patients. If anything, the past 20 years have taught us humility and to approach every patient as an individual.

Immune tolerance, T regulatory cells

The increased prevalence of autoimmune disease (especially thrombocytopenias, arthitis and thyroiditis) seen in DiGeorge syndrome patients suggest a breakdown in immune tolerance. There are numerous mechanisms responsible for immune tolerance in a healthy immune system, however we can broadly divide these into central and peripheral tolerance. Central tolerance has been discussed in an earlier section describing thymocyte development in the cortex and medulla and especially the process of negative selection.

Whether or not the smaller amount of thymic tissue seen in DiGeorge syndrome patients has any impact on this mechanism of negative selection and central tolerance has, to the author's knowledge, not been explored. One very recent study documented a slight decrease of Aire expression in thymi from DiGeorge syndrome patients in the 5-9 month of age period(Marcovecchio et al. 2019), although there was no autoimmunity in any of the 4 evaluated patients and whether they fulfilled the ESID diagnostic criteria for DiGeorge syndrome or only harboured the 22q11.2 deletion is uncertain. This decrease of Aire expression may lead to impaired presentation of tissue-specific antigens on mTECs and lead to generation of autoreactive T cells. For comparison of inter-departmental differences, no thymic tissue is found by surgeons when performing corrective cardiac surgery in infants with DiGeorge syndrome at the author's hospital.

Peripheral tolerance is maintained by several mechanisms, one of which is the activity of Tregs. As mentioned above, developing thymocytes can gain the Treg phenotype (characterized primarily by expression of the FoxP3 transcription factor and high expression of the IL-2 receptor CD25) directly in the thymus. Upon egress into the circulation and peripheral tissues, Tregs suppress and modulate immune response through production of anti-inflammatory cytokines (such as IL-10), granzyme-mediated killing of activated effector T cells, competitive consumption of IL-2 and several other means. Tregs that delineate into a suppressive phenotype already in the thymus are sometimes called natural Tregs (nTregs) or thymic Tregs (tTregs). An alternative means of Treg delineation happens in the periphery when naïve T cells encounter an antigen without the proper secondary stimuli (proinflammatory cytokines, costimulatory molecules on the surface of mature antigen presenting cells etc). These T cells can become induced Tregs (iTregs) or peripheral Tregs (pTregs). Retrospectively distinguishing these origins of Tregs can be problematic due to a lack of specific markers, however some markers have been proposed, including Lap/Garp, Neuropilin-1 and others(Dhamne et al. 2013).

Helios

One among those markers is the transcription factor Helios, a member of the Ikaros family of transcription factors, the expression of which is claimed by some groups to characterize thymic-derived nTregs. Helios is expressed already in the earliest hematopoietic sites in murine embryos and remains present in hematopoietic stem cells in adults, but is restricted to a subset of lymphocytes in the periphery(Hahm et al. 1998; Kelley et al. 1998). In humans, it has been shown to be expressed at highest levels in Tregs. The exact role of Helios remains unclear, but in general terms it seems that it contributes to the overall regulatory phenotype of Tregs. Helios expression upregulates some Treg-related molecules in mice, including GITR, CD103 or GARP(Takatori et al. 2015) and epigenetically silences IL-2 production in Tregs(Baine et al. 2013), supporting the Treg anergic phenotype. Dysfunctional Helios did not hinder Treg suppressive capacity *in vitro* or *in vivo*, but still lead to a slow gradual development of systemic autoimmunity and failure to mediate T follicular regulatory functions(Sebastian et al. 2016). Helios also plays a role at determining the Treg fate, as it binds to the FoxP3 promoter and its knock-down results in downregulation of FoxP3(Getnet et al. 2010).

A number of studies have shown that Helios+ Tregs are functionally, phenotypically and transcriptionally distinct from Helios- Tregs(Thornton et al. 2010, 2019; Yates et al. 2018). Additionally, experiments on germ-free mice have shown that in mice without iTregs, all the Tregs are Helios+, and on the contrary that inoculation with indigenous intestinal *Clostridium* bacteria induces Helios- iTregs in the intestine(Atarashi et al. 2015; Lahtrop et al. 2012). Considering this ontogenic division between nTregs and iTregs and the dysfunction of thymic T cell developmental niche in DiGeorge syndrome patients we set out to evaluate Tregs and Helios expression in DiGeorge syndrome patients.

Tregs in DiGeorge syndrome

Several studies predating 2014 have looked at absolute numbers as well as the proportion of Tregs in CD4 T cells in DiGeorge syndrome patients. The seminal work by Sullivan et al. from 2002 only used proportion of CD25 (the α chain subunit of high affinity IL-2 receptor, characteristic for T regulatory cells) expressing CD4 T cells of all lymphocytes as a readout and not surprisingly found it decreased in DiGeorge syndrome patients, which reflects the overall T cell lymphopenia seen in these patients. Two additional studies used the crucial transcription factor FoxP3 to characterize Tregs and found a decrease of FoxP3+ cells in the CD4 compartment in patients older than 2 years(Ferrando-Martinez et al. 2014; Andrew McLean-Tooke et al. 2008). Tooke et al showed that the proportion of FoxP3+ CD4 T cells increases in healthy but not DiGeorge children, which may suggest that the process of producing additional nTregs during childhood may be thymus dependant. Interestingly, another study found a decrease of Tregs in childhood but not adulthood(Jawad et al. 2011), although no information was

given on what cells were considered as Tregs. The study by Ferrando-Martinez also suggested that DiGeorge syndrome patient Tregs have an activated phenotype, characterized by lack of CD45RA expression, although this may merely reflect the proliferative history and skew towards memory phenotype seen in all DiGeorge T cells. A singular recent study described a modest expansion of Helios- iTregs in the thymic tissue of 3 patients with DiGeorge compared to thymic tissue of healthy controls, but did not comment on Helios expression in the peripheral blood and was limited in size(Marcovecchio et al. 2019). It should also be noted, that several recent studies have cast doubt on the role of Helios as marker of thymic nTregs, showing the possibility of Helios induction in iTregs(Gottschalk, Corse, and Allison 2011), documenting that expression of Helios is associated with cellular activation and proliferation(Akimova et al. 2011) and more. These studies were reviewed by Elkord in 2016(Elkord 2016). The controversy is still ongoing, with studies being published by different groups to this day.

These decreases may play a role in the increased prevalence of autoimmune and atopic disease in DiGeorge syndrome patients. To some extent, the increased homeostatic proliferation of T cells which in patients with thymic dysfunction ensures sufficient numbers of T cells in the setting of decreased thymic output may also contribute to the development of autoimmunity, as increased incidence risk of autoimmune diseases has been documented in children who underwent thymectomy for non-immunologic reasons, compared to surgery controls and healthy population(Gudmundsdottir et al. 2018), even though their proportion of Tregs within CD4 T cells is comparable(Gudmundsdottir et al. 2016).

The results of our study can be seen in the first manuscript attached to this thesis(Klocperk et al. 2014), where we observed preserved proportion of Tregs within CD4 T cells and normal expression of Helios.

Antibody response, B cells, follicular helper T cells

The adaptive immune system is founded by the interaction between T cells and B cells. Both cell types serve a set of very distinct purposes and both require outside help to perform their duties to their fullest extent – be it from each other, or from other specialized cell types of the innate immune system.

The ontogeny of a B cell is particularly intertwined with T cells. For this reason, we and others grew interested in how B cells fare in DiGeorge syndrome.

Bone marrow

Just like T cells, B cells originate in the bone marrow from common lymphoid precursors, however unlike T cells they go through the first part of their development cycle within, rather than travel to a specialized tissue like the thymus. When the decision to become a B cell is reached under the influence

of transcription factors such as E2A or EBF, the pro-B cells start to rearrange their VDJ gene segments in order to ensure a vast array of antigen specificity. First, a section of diversity and joining (D and J) segments is cut out and re-joined by Rag1 and Rag2 enzymes, after which one of the variable (V) segments is attached with all intervening V and D segments being deleted. Additional diversity is added through the activity of terminal deoxynucleotidyl transferase (TdT) which adds non-templated nucleotides to the DNE at the ends of rearranged segments. If this random process is successful and a stable μ heavy chain immunoglobulin is produced, the pro-B cell transitions into a pre-B cell stage and expresses the μ heavy chain on the cell surface together with Vpre-B and λ5 surrogate light chains (invariant, as they haven't been VDJ recombined), forming a pre-B cell receptor (pre-BCR). Should this surface expression be successful, downstream signalling from the pre-BCR halts the VDJ recombination of heavy immunoglobulin chain on the other chromosomal allele, a checkpoint process called "allelic exclusion". The pre-BCR signals then trigger VDJ recombination of light chain genes, first the κ genes and only if no viable κ light chain can be produced, the λ genes. When a light chain is successfully rearranged and expressed together with the μ heavy chain, forming a regular surface IgM BCR, the pre-B cell become an immature B cell and is free to exit the bone marrow. Should any of the checkpoints – expressing a viable pre-BCR or expressing a viable BCR – fail, the cell will die by a programmed cell death. Similarly, if the immature B cell recognizes a bone marrow self-antigen with high avidity, it will either undergo receptor editing, or also suicide in a less involved form of T cell central tolerance / negative selection.

Peripheral blood, secondary lymphoid organs

When immature B cells exit the bone marrow, they become transitional B cells, characterised by high expression of the cell adhesion sialoglycoprotein CD24, cyclic ADP ribose hydrolase CD38 and negativity of the tumor necrosis factor receptor superfamily member, costimulatory checkpoint CD27. They still express high levels of surface IgM, but it should be noted that transitional B cells also express low/intermediate amounts of surface IgD, despite not having undergone class-switch recombination with excision of heavy chain constant segments in the germinal centre (which very rarely leads to a switch into IgD producing cells, due to only a rudimentary switch region present between μ and δ gene segments). IgD expression on transitional B cells is made possible through alternative splicing of the IgM gene mRNA(Geisberger, Lamers, and Achatz 2006). These peripheral transitional B cells are still not capable of fully fledged humoral immune response and overly strong ligation of their BCR will generally result in apoptosis to preserve the body from autoreactive antibodies in an extended central/peripheral tolerance.

Where exactly transitional B cells transition into adulthood as naïve follicular B cells is not entirely clear. Spleen has been proposed to play a significant role, and indeed for some specialized B cell subsets discussed below it is of critical importance. However, since patients who undergo splenectomy are still capable of developing a mature B cell repertoire, it seems that the role of spleen is not irreplaceable. Transitional B cells can be further subdivided into transitional 1, 2 and 3 (T1, 2, 3) based on changing expression of CD10, CD24 and CD38, but this is rarely used in a clinical setting. The survival of transitional B cells – and by extension their further development – is highly dependant on not only tonic BCR signals, but especially serum B cell activating factor (BAFF) levels.

BAFF

BAFF is a soluble cytokine produced by innate immune cells such as neutrophils, macrophages, monocytes, dendritic cells and follicular DCs(Mackay et al. 2003). It's function in supporting B cell development is impressively documented by observation of the immune system in human patients with loss of function mutations in the gene coding BAFFR, who present with almost complete block of B cell development at the immature/transitional stage, overall B cell lymphopenia and agammaglobulinaemia(Smulski and Eibel 2018; Warnatz et al. 2009).

BAFF can bind to several receptors. It can bind to BAFF receptor (BAFFR), to T cell activator and calcium modulating ligand interactor (TACI) and to B cell maturation antigen (BCMA). These receptors all have distinct expression patterns, but ultimately they all support B cell survival and in some cases class switch recombination (TACI).

The supportive biologic activity of BAFF can be a double-edged sword, however, as it's been found to be elevated in autoimmune conditions such as SLE(J. Zhang et al. 2001) or rheumatoid arthritis(Cheema et al. 2001) and can apparently select autoreactive B cells for survival which would otherwise wither(Liu and Davidson 2011). In CVID, BAFF-BAFFR-induced resistance to apoptosis drives the B cell hyperplasia underlying the interstitial lung disease seen in some patients(Maglione et al. 2019).

Marginal zone and marginal zone-like B cells, natural antibodies

Transitional B cells may develop either into naïve follicular B cells which respond to T cell dependent antigens, participate in the germinal centre response leading to production of high affinity class-switched antibodies and are discussed below, or, under specific circumstances, into a unique population of B cells responsive to T cell independent antigens. In mice and also in humans these cells are primarily located in the marginal zone of the spleen, from which comes their name marginal zone B cells, or if located elsewhere, marginal zone-like (MZ-like) B cells. Their surface phenotype includes

high expression of IgM, low IgD and positivity for the memory marker CD27(Cerutti, Cols, and Puga 2013). How transitional B cells delineate into the MZ-like fate is not clear. Signalling through NOTCH2 and the canonical NFκB pathway seems to play a role in mice. Intensive BCR-Btk mediated signaling due to strong BCR ligation supports delineation into naïve follicular B cells, whereas low BCR signaling predisposes cells to develop further along the MZ-like ontogeny(A Cariappa et al. 2000; Annaiah Cariappa et al. 2001; Pillai and Cariappa 2009). In humans, TLR9 recognition of unmethylated bacterial DNA (CpG) promotes development into IgM producing plasma cells, overlapping largely with MZ-like B cells(Capolunghi et al. 2014).

Marginal zone of the spleen, subcapsular sinuses of lymph nodes, subepithelial domes of Peyer's patches and subepithelial regions of tonsils where MZ-like B cells reside are areas of high antigenic load, allowing the MZ-like B cells to sample first-hand a plethora of blood-borne antigens. MZ-like B cells don't require T cell help and are reactive to a specific set of antigens, the so-called T cell independent antigens. These are primarily antigens with repetitive epitopes, the binding of which would result in massive BCR cross-linking, or those which are recognized by innate immune pathogen associated molecular pattern (PAMP) receptors such as TLRs(Kruetzmann et al. 2003). They can also respond to lipid antigens thanks to expression of CD1c. Proving their independence from T cells, MZ-like IgM+IgD+ B cells are preserved – if at reduced counts – in hyper IgM syndrome patients who have mutated CD40L and are not capable of mediating T-B interaction in the germinal centres(Berkowska et al. 2014), whereas the dependence on TLRs is well demonstrated by reduction of MZ-like B cells in patients with mutations in IRAK4, MyD88 and other TLR-related signaling pathways(Weller et al. 2012). Surprisingly, instead of T cells studies have shown interaction between MZ-like B cells and "helper neutrophils" which are a unique subset of neutrophils capable of promoting class switch recombination, somatic hypermutation and antibody production in MZ-like B cells through a mechanism involving BAFF, APRIL and IL-21(Puga et al. 2012).

Thanks to high expression of TLR molecular, specific clonality of BCRs and independence on follicular helper T cells, MZ-like B cells are particularly efficient in rapid response to encapsulated bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae* and others. Indeed, in patients with CVID who couldn't create IgM producing memory B cells, recurrent bacterial pneumonia was more common than in those who had preserved IgM memory B cells(Carsetti et al. 2005).

A unique capability of MZ-like B cells – aside from reacting to polysaccharide antigens without the assistance of T cells – is the production of natural antibodies, which are produced even in the absence of an overt infection. Natural antibodies are generally low affinity and IgM, although particularly in humans may be of IgG or IgA class as well. They target both conserved autoantigens and xenoantigens

and can assist in clearance of apoptotic cells and maintenance of intestinal homeostasis by binding antigens such as the conserved microbacterial α-1,3-galactosylated peptides of intestinal commensal bacteria(Cerutti, Cols, and Puga 2013; Hamanova et al. 2015).

Follicular B cells, follicular helper T cells, germinal center reaction

To produce high-affinity antibodies, memory B cells and long-lived antibody-producing plasma cells, naïve follicular B cells and follicular helper T cells come together in a process called germinal centre reaction which occurs in secondary lymphoid organs(Eibel et al. 2014).

When a professional antigen presenting cell captures an antigen in the blood, lymph or tissue, it carries it through the lymphatic vessels into a local lymph node to present it with costimulatory molecules to naïve T cells in the paracortical T cell zone. Similarly, naïve follicular B cells in lymphoid follicles may recognize the same antigen with their BCR and upon doing so migrate onto the border between T and B zones to engage with the now activated T cells in a mutually beneficial cooperation. This primary response at the border of T and B cell zones is called extrafollicular, as it occurs outside the lymphoid follicles, and during it, the CD40L-CD40 interaction between the T cells and B cells induces several fold proliferation of all interacting cells, creation of short-lived low-affinity IgM-producing plasma cells and immunoglobulins, some of which may already be class-switched into non-IgM classes (though this is rare and somatic hypermutation is not yet present to allow for high-affinity antibodies).

Within a week of primary encounter with the antigen, some of the activated B cells migrate back into the lymphoid follicle and start replicating rapidly in clusters of small B cells called centroblasts which form the dark zone of a germinal center. Their progeny migrates outwards and as centrocytes enter the light zone, where they interact with follicular helper T cells and follicular dendritic cells. Here, only those cells with highest affinity can obtain large amounts of antigen from the follicular dendritic cells and in turn present it to the follicular helper T cells(Batista, Iber, and Neuberger 2001). Those that do receive survival signals through CD40L-CD40 and IL-21 and can return for re-expansion to the dark zone, those that do not are assisted in programmed cell death through Fas. Over the course of many cycles this process of migrating back and forth between dark and light zones gradually increases the overall affinity of produced antibodies(Bannard et al. 2013). The increasing affinity is enabled by somatic hypermutation of the immunoglobulin variable regions. Both somatic hypermutation and immunoglobulin class switch rely on activation induced deaminase (AID), an enzyme whose absence will also cause arrest of B cell development before the germinal centre reaction and thus result in the clinical and laboratory presentation of a hyper IgM syndrome(Revy et al. 2000).

Which gene segments will be excised during class-switch recombination depends largely on the cytokine milieu in the germinal center. Follicular helper T cells are particularly responsible for the

direction in which the response will develop, as they are potent producers of not only the IL-21 necessary for B cell proliferation, but also cytokines inducing class switching into IgG, IgE or IgA. In fact, follicular helper T cells diverge into several lineages that correspond to their effector T cell counterparts in both cytokine production and transcriptomic profile – IFNγ producing cTFH1, IL-4 producing cTFH2 and IL-17 producing cTFH17(Morita et al. 2011). In turn, IL-4 supports switching to IgE, TGFβ can support switching to IgA or IgG2 and while some earlier reports suggested that IFNγ can support switching to IgG2 in mice(Kawano, Noma, and Yata 1994), this has not been replicated in humans. In fact, it appears that IFNγ producing cTFH1 are not very efficient B cell helpers, but can in fact drive autoimmunity with rampant IFN signature(Unger et al. 2017).

Interestingly, a small amount of follicular helper T cells can be detected circulating in the peripheral blood. These circulating follicular helper T cells (cTFHs) carry the classical follicular helper T cell phenotype, featuring expression of CXCR5 enabling them to home into the lymphoid follicles after the CXCL13 gradient. Some studies have included expression of PD-1 and ICOS(Bauquet et al. 2009) or capability of IL-21 production in their definitions and there is not unified approach past the positivity for CXCR5.

cTFHs have been examined in many primary immunodeficiencies tied to aberrant production of antibodies(C. S. Ma et al. 2015) and in several autoimmune disorders such as SLE, Sjögren syndrome or rheumatoid arthritis(J. Ma et al. 2012; Szabo et al. 2013; X. Zhang et al. 2015). While autoimmunity generally sees cTFHs increased(Ueno 2016), we tend to observe a reduction of cTFHs in primary immunodeficiencies with impaired antibody response or T cell differentiation. Of note, patients with X-linked agammaglobulinaemia due Btk mutations in whom T cells should be unaffected have actually very significantly decreased cTFHs, presumably due to the missing B cells required for a functional germinal center response and delineation of follicular helper T cells. Similar reduction is seen in patients with IL-10R and CD40L mutations(C. S. Ma et al. 2015).

B cells and follicular helper T cells in diGeorge syndrome

The importance of T cells for establishment of long-term protective antibody response and generation of high affinity specific antibodies vital for vaccination and defence against infections understandably lead to interest in B cells and germinal center response in DiGeorge syndrome patients.

Earlier studies have shown normal numbers of B cells and an intriguing trend from hypogammaglobulinaemia in childhood which may even warrant immunoglobulin replacement therapy(Patel et al. 2012), towards hypergammaglobulinaemia in adolescence(Junker et al. 1995; Müller et al. 1989). Response to vaccination was poor in one study with 55% of patients not responding to a pneumococcal polysaccharide vaccine(Gennery et al. 2002) and another which found impaired

response to the influenza vaccine(Jawad et al. 2011), however others have shown good post-vaccination antibodies(Müller et al. 1989). When tested, natural antibodies against BSA were positive in 6/13 partial DiGeorge syndrome patients, which is similar to their prevalence in general population(Mogues et al. 2005), suggesting preserved MZ-like function. The trend from low IgG in infancy and early childhood into high IgG in adolescence and adulthood has been documented repeatedly and may reflect impaired T cell help concomitant with most severe T cell lymphopenia in early ages, with gradual normalization of T cell counts and increased stimulation of IgG production through recurrent infections over the subsequent years(Gennery et al. 2002; Jawad et al. 2001; Klocperk et al. 2015; Šedivá et al. 2005).

The advent of flow cytometry in the early 2000s allowed higher resolution for determining developmental stages of B cells, such as CD27-IgM+IgD+ naïve B cells, CD27-24hi38hi transitional B cells, CD27+IgD+ MZ-like B cells or CD27+IgD- switched memory B cells. First studies making use of this technique have shown a decrease of all memory B cells in DiGeorge syndrome, both class-switched and not(Finocchi et al. 2006; Andrew McLean-Tooke et al. 2008; Zemble et al. 2010). Naïve B cells were low in children but not in adults(Zemble et al. 2010). A study by Jawad et al from 2011 also suggests a decrease of innate-like B1 cells bearing expression of CD5 in DiGeorge adults(Jawad et al. 2011), however the B1 population is generally not well defined in humans and should be interpreted with caution.

The current frontier of unravelling the T-B cooperation in DiGeorge syndrome is the population of cTFHs. Until recently those have not been looked at in DiGeorge, until a paper in 2016 by Derfalvi et al, who found increased CXCR5+ICOS+ cTFHs in both children and adult patients(Derfalvi et al. 2016).

Our findings describing the humoral and germinal center reactions, B cell subpopulations and cTFHs in patients with DiGeorge syndrome followed at our department are summarized in the papers attached to this thesis(Klocperk et al. 2015, 2018), where we document impaired B cell maturation despite preserved population of cTFHs and a decrease of MZ-like B cells with low natural antibodies.

Therapeutic options

In general, therapeutic management of patients with DiGeorge syndrome changes with age. In early infancy the focus is on treating the cardiac anomalies, potential biochemical aberrations secondary to hypoparathyroidism and ruling out or treating severe immunodeficiency. Childhood and exposure of patients to their peers sees the rise of recurrent infections as the immune memory struggles to establish, so careful approach to vaccination and/or immunomodulation or supportive therapy by an immunologist is necessary. Additionally, logopaedic assistance may be useful for children with

substantial velopharyngeal insufficiency to facilitate speech development. In later life, the increased prevalence of allergies may bring further necessity of immunological/allergological follow-up, and due to possible difficulties in school and increased risk of developing schizophrenia, psychological or even psychiatrical follow-up or treatment may be useful(Bassett, McDonald-McGinn, and Devriendt 2011). Despite these general tenets, the therapeutic and follow-up approach to patients with DiGeorge syndrome is highly individual and based on particular needs of each patient.

Therapeutic options for the immunodeficiency seen in DiGeorge syndrome range from careful follow-up all the way to hematopoietic stem cell transplantation, T cell transfer or thymic transplantation. All patients should be followed by a clinical immunologist at a centre capable of evaluation both cellular and humoral immune parameters, with access to other specialists based on other complications present in each patient. The approach is individualized based on severity of immunodeficiency. In patients with mild phenotype and only slightly decreased T cells, delaying vaccination with live vaccines until CD4 T cells rise above 400 cells/mm^3 and careful follow-up and monitoring may be sufficient. More commonly, however, more severe T cell lymphopenia requires prophylactic administration of antibiotics, usually sulfamethoxazole + trimethoprim twice weekly. In patients with hypogammaglobulinaemia, intravenous or subcutaneous immunoglobulin substitution is advised, usually starting at 400 mg/kg monthly and further titrated based on immunoglobulin trough levels.

For most patients with partial DiGeorge syndrome, these conservative approaches offer the best cost/benefit ratio. For complete DiGeorge syndrome, however, the risk of life-threatening infection is too high and curative approaches must be considered with urgency.

Historically, transplantation of hematopoietic stem cells was the only option for treatment of complete DiGeorge syndrome. Even though the function of the bone marrow is preserved in DiGeorge patients, the transplantation also transfers a portion of adult post-thymic T cells, which can then homeostatically proliferate even in the complete absence of thymus. The long-term outcome is poor, however, at just around 50% five year survival, ranging from 33% for a matched unrelated donor to 60% for a matched sibling donor(McGhee, Lloret, and Stiehm 2009). PBMCs have also been used as a source material with middling success.

Transplantation of thymic tissue would solve the developmental crisis of T cells while preserving the bone marrow. Post-natal transplantation of foetal thymic tissue has been used for a handful of cases(Cleveland et al. 1968; Mayumi et al. 1989), but in some of them the immune reconstitution may have been due to an already existing partially preserved T cell population, and the overall outcome was poor with numerous cases of lymphoproliferative disease after transplantation(Borzy et al. 1979). In 1999, a seminal paper published by Markert et al described a technique for transplantation of *in*

vitro cultured post-natal thymic tissue, which lead to immune reconstitution in 3/5 patients with no signs of GVHD(M L Markert et al. 1999). In the following 10 years, over 60 patients have been transplanted with an overall five year survival rate of around 70%(M. Louise Markert, Devlin, and McCarthy 2010). Recently this method has also been established in Europe with over 90% survival rate(E. G. Davies et al. 2017). First patient from the Czech Republic to undergo thymic transplantation is a girl with complete DiGeorge syndrome followed at the University Hospital in Motol, Prague, whose thymus was transplanted at the Great Ormond Street in London in July 2017 and who, two years after transplantation, is showing solid T cell reconstitution in complete absence of autoimmunity or GVHD (Klocperk and Šedivá 2019, submitted to Alergie).

AIMS OF THIS THESIS

As documented extensively in the introduction, DiGeorge syndrome is formed by a phenotypically rich spectrum of patients with immunologic, cardiologic, endocrinologic and psychologic problems. In the past 50 years, its unique pathophysiology with absence or dysplasia of thymus has taught us a lot about the function of the organ and the plasticity of human immune system, however, many important questions about the disease's pathophysiology remain unanswered.

Thus, the aims of this doctoral project were to:

1. Explore the impact thymic dysplasia and deletion of 22q11.2 has on development of T cells in patients with DiGeorge syndrome
2. Assess the generation of regulatory T cells (Tregs) in DiGeorge syndrome patients and healthy donors and to distinguish between thymic-derived and peripheral-induced Tregs using the expression of transcription factor Helios
3. Evaluate the effect of thymus-induced T cell lymphopenia and impaired T cell maturation on B cells and humoral immune response
4. Separately quantify T cell-dependent and T cell-independent humoral immunity in DiGeorge syndrome
5. Explore the germinal center response in DiGeorge syndrome with focus on T follicular helper cells, as potential cause of autoimmunity and hypergammaglobulinaemia

PUBLISHED PAPERS

Pertaining directly to this thesis

Klocperk, A., Grecová, J., Sišmová, K., Kayserová, J., Froňková, E., Sedivá, A., … Šedivá, A. (2014). Helios Expression in T-regulatory Cells in Patients with di George Syndrome. *Journal of Clinical Immunology*, *34*(7), 864–870. https://doi.org/10.1007/s10875-014-0071-y

Klocperk, A., Mejstříková, E., Kayserová, J., Kalina, T., & Šedivá, A. (2015). Low marginal zone-like B lymphocytes and natural antibodies characterize skewed B-lymphocyte subpopulations in del22q11 DiGeorge patients. *Clinical Immunology*, *161*(2), 144–149.

Klocperk, A., Paračková, Z., Bloomfield, M., Rataj, M., Pokorný, J., Unger, S., … Šedivá, A. (2018). Follicular Helper T Cells in DiGeorge Syndrome. *Frontiers in Immunology*, *9*(July), 1–9. https://doi.org/10.3389/fimmu.2018.01730

Pertaining to other projects

Froňková, E., Klocperk, A., Svaton, M., Nováková, M., Kotrova, M., Kayserova, J., … Anna, S. (2014). The TREC/KREC assay for the diagnosis and monitoring of patients with DiGeorge syndrome. *PLoS ONE*, *9*(12), 1–13. https://doi.org/10.1371/journal.pone.0114514

Milota, T., Šumník, Z., Obermannová, B., Králíčková, P., Vondrák, K., Klocperk, A., … Šedivá, A. (2016). Negativity for Specific Autoantibodies in Patients with Type 1 Diabetes That Developed on a Background of Common Variable Immunodeficiency. *International Archives of Allergy and Immunology*, *168*(3), 197–204. https://doi.org/10.1159/000441723

Bloomfield, M., Kanderová, V., Paračková, Z., Vrabcová, P., Svatoň, M., Froňková, E., … Šedivá, A. (2018). Utility of ruxolitinib in a child with chronic mucocutaneous candidiasis caused by a novel STAT1 gain-of-function mutation. *Journal of Clinical Immunology*, *38*(5), 589–601. https://doi.org/10.1007/s10875-018-0519-6

Ponsford, M. J., Klocperk, A., Pulvirenti, F., Dalm, V. A. S. H., Milota, T., Cinetto, F., … Jolles, S. (2018). Hyper IgE in the Allergy Clinic- when is it Primary Immunodeficiency? *Allergy*. https://doi.org/10.1111/all.13578

Ponsford, M. J., Rae, W., & Klocperk, A. (2018). What's new in HIES? Recent insights from the interface of primary immune deficiency and atopy. *Current Opinion in Allergy and Clinical Immunology*, *18*, 445–452.

Králíčková, P., Milota, T., Litzman, J., Malkušová, I., Jílek, D., Petanová, J., … Šedivá, A. (2018). CVID-associated tumors – Czech nationwide study focused on epidemiology, immunology and genetic background in a cohort of patients with CVID. *Frontiers in Immunology*.

Kanderova, V., Grombirikova, H., Zentsova, I., Reblova, K., Klocperk, A., Fejtkova, M., … Freiberger, T. (2018). Lymphoproliferation, immunodeficiency and early-onset inflammatory bowel disease associated with a novel mutation in Caspase 8. *Haematologica*, *103*. https://doi.org/10.3324/haematol.2018.201673

SUMMARY OF PUBLISHED WORK (ENG)

In this thesis I discuss the complexity and history of primary immunodeficiencies and I describe the causes, clinical phenotype, immunological findings and treatment options of DiGeorge syndrome.

In the publications attached above, we describe the following findings:

1. Absolute and relative T cell counts are reduced in patients with partial DiGeorge syndrome in all age groups. The relative percentage but not absolute counts of memory CD4 T cells are increased, showing decreased thymic output and extensive history of T cell proliferation.
2. Regulatory T cells form a comparable proportion of CD4 T cells in DiGeorge syndrome, with progressive decrease identical to that of healthy controls. Thus, the increased prevalence of autoimmunity seen in DiGeorge syndrome is not due to low Tregs but may be caused by their faulty function of other reasons.
3. There is no difference in proportion of Helios+ Tregs in partial DiGeorge syndrome patients, suggesting that thymic development of Tregs is unimpeded, or that Helios is not a good marker of thymic Tregs.
4. DiGeorge syndrome patients have preserved absolute B cell counts, but increased proportion of naïve B cells (CD27-IgD+) and decreased proportion of class-switched memory B cells (CD27+IgD-). The overall distribution of B cell subsets is disturbed in DiGeorge syndrome patients. The inability to develop class-switched memory B cells and long-term humoral immune protection appears in the 5-10 years age group, suggesting that exposure to antigens in early childhood fails to promote the germinal center response in DiGeorge syndrome patients.
5. The increase in naïve B cells is reflected also in increased levels of serum BAFF, which is required for naïve B cell survival. The heightened serum BAFF levels may play a role in the development of autoimmune phenomena seen in patients.
6. DiGeorge syndrome patients fail to upregulate MZ-like B cells (CD27+IgD+) conferring T-independent humoral response, and this is reflected in starkly decreased natural anti-α-Gal antibodies. This failure may cause the increased susceptibility to infections with encapsulated bacteria seen in patients.
7. Despite the germinal center reaction failure, circulating follicular helper T cells are increased in DiGeorge syndrome patients, however they do not correlate with proportion of class-switched memory B cells or serum IgG. Similarly, cTFH counts do not differentiate between patients with and without allergy or autoimmune thrombocytopenia. Functions and detailed subsets of cTFHs DiGeorge syndrome are subject of ongoing investigation.

SOUHRN PUBLIKOVANÉ PRÁCE (CZ)

V této dizertační práci diskutuji komplexitu patofyziologie, imunologických a klinických manifestací primárních imunodeficiencí a popisuji příčiny, klinický fenotyp, imunologické nálezy a možnosti léčby DiGeorgova syndromu.

Ve výše přiložených publikacích popisujeme následující výsledky:

1. Absolutní počty i relativní proporce T lymfocytů jsou sníženy ve všech věkových kategoriích pacientů s parciálním syndromem DiGeorge. Percentuální zastoupení ale nikoliv absolutní počty paměťových CD4 T lymfocytů jsou zvýšeny, což dokazuje sníženou funkci thymu a proliferační historii T lymfocytů.
2. Proporce Tregů z CD4 T lymfocytů i její postupný pokles s věkem je u pacientů srovnatelný se zdravými kontrolami. Tudíž zvýšená prevalence autoimunitních onemocnění u pacientů se syndromem DiGeorge není způsobena nízkým počtem Tregů, ale na vině může být jejich dysfunkce či jiné důvody.
3. Nepozorujeme rozdíl v zastoupení Helios+ Tregů mezi DiGeorge pacienty a zdravou populací, což značí, že vývoj Tregů v thymu není postižen, či že Helios není dobrým znakem thymických Tregů.
4. Pacienti se syndromem diGeorge mají normální počty B lymfocytů, ale celkové rozložení subpopulací B lymfocytů je u pacientů abnormální. Nacházíme zvýšenou proporci naivních (CD27-IgD+) a sníženou proporci přesmyklých paměťových (CD27+IgD-) B lymfocytárních subpopulací. Neschopnost pacientů se syndromem DiGeorge vytvořit přesmyklé paměťové B lymfocyty a tudíž zajistit dlouhotrvající protilátkovou obranyschopnost organismu se objevuje mezi 5. a 10. rokem věku, tedy po expozici komunálním patogenům v dětských kolektivech.
5. Zvýšení naivních B lymfocytů se odráží též ve vysokých sérových koncentrací BAFF, jenž je nepostradatelný pro jejich přežití. Toto zvýšení BAFF může vést k rozvoji autoimunitních komplikací, které vídáme u části pacientů.
6. U pacientů nedochází mezi 2. a 5. rokem věku ke zvýšení proporce MZ-like B lymfocytů, které vidíme u zdravých kontrol. Tyto lymfocyty zajišťují T-nezávislou humorální odpověď a produkují přirozené protilátky jako například anti-α-Gal, jejichž hladiny mají pacienti v séru snížené. Tento nedostatek MZ-like B lymfocytů může vést ke zvýšené náchylnosti k infekcím opouzdřenými bakteriemi.
7. Přes zjevně nefunkční reakci zárodečného centra, cirkulující pomocné T lymfocyty jsou u pacientů se syndromem DiGeorge zvýšeny. Jejich počty však nekorelují s proporcí přesmyklých paměťových B lymfocytů, ani se sérovými hladinami IgG. Cirkulující pomocné T lymfocyty se

též neliší mezi skupinou pacientů s/bez alergií a s/bez trombocytopenií. Funkce a subpopulace Tfh lymfocytů jsou předmětem probíhajícího výzkumu.

FUTURE PLANS AND IMMUNODEFICIENCIES BEYOND DIGEORGE SYNDROME

In the months following the publication of the last paper describing follicular helper T cells in DiGeorge syndrome we have become interested in what effect does the thymic dysplasia in DiGeorge syndrome, thymectomy performed in patients with congenital heart disease and thymectomy in patients with myasthenia gravis or Good's syndrome have on T cell lineages (including IFNγ-producing Th1, IL-17-producing Th17 or IL-4-producing Th2) and T cell senescence and exhaustion.

To explore this, we are setting up collaboration with the Children's Heart Centre of the Motol hospital and the Department of Surgery to obtain blood and thymic tissue of these patients. We will employ high-parametric flow cytometry or mass cytometry with subsequent multidimensional analysis and dimensionality reduction methods such as tSNE, FlowSOM and others, as well as RNA sequencing and systems biology approach.

The knowledge, experience and methodology already developed as part of this project is being translated into other projects planned or ongoing at our department. Their full description would exceed the scope of this thesis, but the author has nevertheless been and continues to be involved in several of them, as documented by the list of published papers.

To support the involvement in multiple projects, our research group has received a national grant aimed at the functional evaluation, monitoring and therapy for newly diagnosed patients with primary immunodeficiency and immune dysregulation. This approach is especially helpful in those patients whose genes bear variants of unknown significance, such as for example in one patient suffering from generalized lymphadenopathy caused by caspase 8 deficiency(Kanderova et al. 2018), and those where specific targeted therapy can be considered, such as for example in a patient with chronic mucocutaneous candidiasis caused by STAT1 gain-of-function mutation(Bloomfield et al. 2018). Our group thus leads a nation-wide effort in disseminating high-level care to patients suffering from multitude of conditions from across the Czech Republic and collaborates with other centers at both national and international level(Králíčková et al. 2018).

Our international collaboration and the author's laboratory rotation at the Centre for Chronic Immunodeficiency in Freiburg in 2018 expanded our experience with CVID and lead to participation on several ongoing projects. In particular these include the associations and pathophysiology of enteropathy in CVID, the phenotype and features of exhaustion in CD8 T cells in CVID and the role of follicular CXCR5+ CD8 T cells in CVID lymphadenopathy. At least two manuscripts describing our findings are currently being prepared for submission. The author has also co-authored two international review papers on primary atopic diseases and hyper-IgE syndromes(Ponsford et al. 2018;

Ponsford, Rae, and Klocperk 2018), some of which feature among the patients followed at our department. Further projects currently being launched or already ongoing include immunophenotyping and monitoring of the metabolic status of CVID patients, evaluation of the effect of biologic therapy in autoimmune diseases, immunobiology of type 1 diabetes and prediction of its onset and others.

Looking to the future, the author hopes to further utilize and expand his wet lab and bioinformatics skills, which along with the clinical perspective of an actively practicing physician should serve as a solid and rational basis for impactful clinical immunology research.

REFERENCES

Aaltonen, Johanna et al. 1997. "An Autoimmune Disease, APECED, Caused by Mutations in a Novel Gene Featuring Two PHD-Type Zinc-Finger Domains." *Nature Genetics* 17(4): 399–403.

Abramson, Jakub, and Yael Goldfarb. 2016. "AIRE: From Promiscuous Molecular Partnerships to Promiscuous Gene Expression." *European Journal of Immunology* 46(1): 22–33.

Akimova, Tatiana et al. 2011. "Helios Expression Is a Marker of T Cell Activation and Proliferation." *PloS one* 6(8): e24226.

Alper, Chester A. et al. 2003. "Immunoglobulin Deficiencies and Susceptibility to Infection among Homozygotes and Heterozygotes for C2 Deficiency." *Journal of Clinical Immunology* 23(4): 297–305.

Ammann, A J et al. 1982. "The DiGeorge Syndrome and the Fetal Alcohol Syndrome. - PubMed - NCBI." *American Journal of Diseases of Children* 136(10): 906–8.

Atarashi, Koji et al. 2015. "Induction of Colonic Regulatory T Cells by Indigenous Clostridium Species." *Science* 27(3): 320–31.

Bachmann, M. F. et al. 2014. "Functional Properties and Lineage Relationship of CD8+ T Cell Subsets Identified by Expression of IL-7 Receptor and CD62L." *The Journal of Immunology* 175(7): 4686–96.

Baine, I et al. 2013. "Helios Induces Epigenetic Silencing of Il2 Gene Expression in Regulatory T Cells." *Journal of Immunology* 190(3): 1008–16.

Bannard, Oliver et al. 2013. "Germinal Center Centroblasts Transition to a Centrocyte Phenotype According to a Timed Program and Depend on the Dark Zone for Effective Selection." *Immunity* 39(5): 912–24.

Bassett, Anne S, Donna M McDonald-McGinn, and Koen Devriendt. 2011. "Practical Guidelines for Managing Patients with 22q11." *Journal of Pediatrics* 159(2): 1–17.

Batista, F. D., D. Iber, and M. S. Neuberger. 2001. "B Cells Acquire Antigen from Target Cells after Synapse Formation." *Nature* 411(6836): 489–94.

Bauquet, Aurelie T et al. 2009. "Costimulatory Molecule ICOS Plays a Critical Role in the Development of TH -17 and Follicular T-Helper Cells by Regulating c- Maf Expression and IL-21 Production." *Nature Immunology* 10(2): 167–75.

Berkowska, Magdalena A et al. 2014. "Human Memory B Cells Originate from Three Distinct Germinal Center-Dependent and -Independent Maturation Pathways." *Blood* 118(8): 2150–58.

Blackburn, C. C. et al. 2002. "The Nu Gene Acts Cell-Autonomously and Is Required for Differentiation of Thymic Epithelial Progenitors." *Proceedings of the National Academy of Sciences* 93(12): 5742–46.

Bloomfield, Markéta et al. 2018. "Utility of Ruxolitinib in a Child with Chronic Mucocutaneous Candidiasis Caused by a Novel STAT1 Gain-of-Function Mutation." *Journal of Clinical Immunology* 38(5): 589–601.

Borzy, M S et al. 1979. "Fatal Lymphoma after Transplantation of Cultured Thymus in Children with Combined Immunodeficiency Disease." *New England Journal of Medicine* 301(11): 565–68.

Bruton, Ogden C. 1952. "AGAMMAGLOBULINEMIA." *Pediatrics* 9(6).

Burn, J. 1999. “Closing Time for CATCH22.” *Journal of Medical Genetics* 36(10): 737–38.

Capolunghi, F. et al. 2014. “CpG Drives Human Transitional B Cells to Terminal Differentiation and Production of Natural Antibodies.” *The Journal of Immunology* 180(2): 800–808.

Cariappa, A, H C Liou, B H Horwitz, and S Pillai. 2000. “Nuclear Factor Kappa B Is Required for the Development of Marginal Zone B Lymphocytes.” *The Journal of experimental medicine* 192(8): 1175–82.

Cariappa, Annaiah et al. 2001. “The Follicular versus Marginal Zone B Lymphocyte Cell Fate Decision Is Regulated by Aiolos, Btk, and CD21.” *Immunity* 14(5): 603–15.

Carlson, C. et al. 1997. “Molecular Definition of 22q11 Deletions in 151 Velo-Cardio-Facial Syndrome Patients.” *The American Journal of Human Genetics* 61(3): 620–29.

Carsetti, Rita et al. 2005. “The Loss of IgM Memory B Cells Correlates with Clinical Disease in Common Variable Immunodeficiency.” *Journal of Allergy and Clinical Immunology* 115(2): 412–17.

Casanova, Jean-Laurent. 2015. “Severe Infectious Diseases of Childhood as Monogenic Inborn Errors of Immunity.” *Proceedings of the National Academy of Sciences of the United States of America* 112(51): E7128-37.

Cerutti, Andrea, Montserrat Cols, and Irene Puga. 2013. “Marginal Zone B Cells: Virtues of Innate-like Antibody-Producing Lymphocytes.” *Nature Reviews Immunology* 13(2): 118–32.

Cheema, Gurtej S, Viktor Roschke, David M Hilbert, and William Stohl. 2001. “Elevated Serum B Lymphocyte Stimulator Levels in Patients With Systemic Immune – Based Rheumatic Diseases.” *Arthritis & Rheumatism* 44(6): 1313–19.

Cheung, Evelyn Ning Man et al. 2014. “Prevalence of Hypocalcaemia and Its Associated Features in 22q11·2 Deletion Syndrome.” *Clinical Endocrinology* 81(2): 190–96.

Chinen, Javier et al. 2003. “Long-Term Assessment of T-Cell Populations in DiGeorge Syndrome.” *Journal of Allergy and Clinical Immunology* 111(3): 573–79.

Cleveland, W.W., B.J. Fogel, W.T. Brown, and H.E.M. Kay. 1968. “FŒTAL THYMIC TRANSPLANT IN A CASE OF DiGEORGE’S SYNDROME.” *The Lancet* 292(7580): 1211–14.

Coberly, Suzanne, Edward Lammer, and Mouied Alashari. 1996. “Retinoic Acid Embryopathy: Case Report and Review of Literature.” *Pediatric Pathology and Laboratory Medicine* 16(5): 823–36.

Cordier, André C., and Stanislas M. Haumont. 1980. “Development of Thymus, Parathyroids, and Ultimo-branchial Bodies in NMRI and Nude Mice.” *American Journal of Anatomy* 157(3): 227–63.

Cunningham-Rundles, Charlotte. 2012. “The Many Faces of Common Variable Immunodeficiency.” *Hematology. American Society of Hematology. Education Program*: 301–5.

Davies, E Graham et al. 2017. “Thymus Transplantation for Complete Digeorge Syndrome: European Experience.” *Journal of Allergy and Clinical Immunology*.

Davies, K, E R Stiehm, P Woo, and K J Murray. 2001. “Juvenile Idiopathic Polyarticular Arthritis and IgA Deficiency in the 22q11 Deletion Syndrome.” *The Journal of rheumatology* 28(10): 2326–34.

Derfalvi, Beata et al. 2016. “B Cell Development in Chromosome 22q11.2 Deletion Syndrome.” *Clinical Immunology* 163: 1–9.

Dhamne, Chetan et al. 2013. “Peripheral and Thymic Foxp3+ Regulatory T Cells in Search of Origin, Distinction, and Function.” *Frontiers in Immunology* 4(August): 1–11.

DiGeorge, A M, H W Lischner, C Dacou, and J B Arey. 1967. “Absence of Thymus.” *Lancet* 289(7504).

DiGeorge, Angelo. 1965. “Discussion of Cooper et. Al.” *Journal of Pediatrics* 67(5): 907.

Digilio, Maria Cristina, Bruno Marino, Roberto Formigari, and Aldo Giannotti. 1995. “Maternal Diabetes Causing DiGeorge Anomaly and Renal Agenesis.” *American Journal of Medical Genetics* 55(4): 513–14.

Downie, A. W. 1951. “Jenner’s Cowpox Inoculation.” *British Medical Journal* 2(4726): 251–56.

Driscoll, D A et al. 1993. “Prevalence of 22q11 Microdeletions in DiGeorge and Velocardiofacial Syndromes: Implications for Genetic Counselling and Prenatal Diagnosis.” *Journal of medical genetics* 30(10): 813–17.

Driscoll, D a, M L Budarf, and B S Emanuel. 1992. “A Genetic Etiology for DiGeorge Syndrome: Consistent Deletions and Microdeletions of 22q11.” *American journal of human genetics* 50(5): 924–33.

Eberle, P. et al. 2009. “Persistent Low Thymic Activity and Non-Cardiac Mortality in Children with Chromosome 22q11.2 Microdeletion and Partial DiGeorge Syndrome.” *Clinical and Experimental Immunology* 155(2): 189–98.

Eibel, Hermann et al. 2014. “B Cell Biology: An Overview.” *Current allergy and asthma reports* 14: 434.

Elkord, Eyad. 2016. “Helios Should Not Be Cited as a Marker of Human Thymus-Derived Tregs. Commentary: Helios+ and Helios− Cells Coexist within the Natural FOXP3+ T Regulatory Cell Subset in Humans.” *Frontiers in Immunology* 7.

Ferrando-Martinez, Sara et al. 2014. “Low Thymic Output, Peripheral Homeostasis Deregulation, and Hastened Regulatory T Cells Differentiation in Children with 22q11.2 Deletion Syndrome.” *The Journal of Pediatrics* 164(4): 882–89.

Finocchi, A et al. 2006. “Humoral Immune Responses and CD27+ B Cells in Children with DiGeorge Syndrome (22q11.2 Deletion Syndrome).” *Pediatric Allergy and Immunology* 17(5): 382–88.

Fudenberg, H H et al. 1970. “Classification of the Primary Immune Deficiencies: WHO Recommendation.” *New England Journal of Medicine* 283(12): 656–57.

Geisberger, Roland, Marinus Lamers, and Gernot Achatz. 2006. “The Riddle of the Dual Expression of IgM and IgD.” *Immunology* 118(4): 429–37.

Gennery, A R et al. 2002. “Antibody Deficiency and Autoimmunity in 22q11.2 Deletion Syndrome.” *Archives of Disease in Childhood* 86: 422–25.

Germain, Ronald N. 2002. “T-Cell Development and the CD4-CD8 Lineage Decision.” *Nature Reviews Immunology* 2(5): 309–22.

Getnet, Derese et al. 2010. “A Role for the Transcription Factor Helios in Human CD4 + CD25 + Regulatory T Cells.” *Molecular Immunology* 47(7–8): 1595–1600.

Giannotti, A. et al. 1994. “Cayler Cardiofacial Syndrome and Del 22q11: Part of the CATCH22 Phenotype.” *American Journal of Medical Genetics* 53(3): 303–4.

Giraud, M. et al. 2011. “Aire Unleashes Stalled RNA Polymerase to Induce Ectopic Gene Expression in Thymic Epithelial Cells.” *Proceedings of the National Academy of Sciences* 109(2): 535–40.

Goldblatt, D., and A. J. Thrasher. 2000. “Chronic Granulomatous Disease.” *Clinical and Experimental Immunology* 122(1): 1–9.

Göran, J et al. 2005. “Hereditary C2 Deficiency in Sweden.” *Medicine* 84(1): 23–34.

Gottschalk, R. A., E. Corse, and J. P. Allison. 2011. “Expression of Helios in Peripherally Induced Foxp3+ Regulatory T Cells.” *The Journal of Immunology* 188(3): 976–80.

Gudmundsdottir, Judith et al. 2016. “Early Thymectomy Leads to Premature Immunologic Ageing: An 18-Year Follow-Up.” *Journal of Allergy and Clinical Immunology*.

———. 2018. “Long-Term Clinical Effects of Early Thymectomy: Associations with Autoimmune Diseases, Cancer, Infections, and Atopic Diseases.” *Journal of Allergy and Clinical Immunology* 141(6): 2294–97.

Hahm, Kyungmin et al. 1998. “Helios, a T Cell-Restricted Ikaros Family Member That Quantitatively Associates with Ikaros at Centromeric Heterochromatin.” *Genes and Development* 12(6): 782–96.

Hamanova, Marketa et al. 2015. “Anti-Gal IgM, IgA and IgG Natural Antibodies in Childhood.” *Immunology Letters* 164(1): 40–43.

Hill, M A. 2019. “Thymus Histology 06.Jpg.” *Embryology*. https://embryology.med.unsw.edu.au/embryology/index.php/File:Thymus_histology_06.jpg.

Huber, F X et al. 2011. “Aire Regulates the Transfer of Antigen from MTECs to Dendritic Cells for Induction of Thymic Tolerance.” *Blood* 118(9): 2462–72.

Jawad, Abbas F et al. 2011. “A Prospective Study of Influenza Vaccination and a Comparison of Immunologic Parameters in Children and Adults with Chromosome 22q11.2 Deletion Syndrome (Digeorge Syndrome/Velocardiofacial Syndrome).” *Journal of Clinical Immunology* 31(6): 927–35.

Jawad, Abbas F, D M McDonald-Mcginn, E Zackai, and K E Sullivan. 2001. “Immunologic Features of Chromosome 22q11.2 Deletion Syndrome (DiGeorge Syndrome/Velocardiofacial Syndrome).” *The Journal of pediatrics* 139(5): 715–23.

Jongmans, M C J et al. 2006. “CHARGE Syndrome: The Phenotypic Spectrum of Mutations in the CHD7 Gene.” *Journal of Medical Genetics* 43(4): 306–14.

Junge, Sonja et al. 2007. “Correlation between Recent Thymic Emigrants and CD31+ (PECAM-1) CD4+ T Cells in Normal Individuals during Aging and in Lymphopenic Children.” *European Journal of Immunology* 37(11): 3270–80.

Junker, A K, D A Driscoll, Immunological Diseases, and British Columbia. 1995. “Humoral Immunity in DiGeorge Syndrome.” *The Journal of pediatrics* 127(2): 231–37.

Jyonouchi, S. et al. 2009. “CHARGE (Coloboma, Heart Defect, Atresia Choanae, Retarded Growth and Development, Genital Hypoplasia, Ear Anomalies/Deafness) Syndrome and Chromosome 22q11.2 Deletion Syndrome: A Comparison of Immunologic and Nonimmunologic Phenotypic Features.” *Pediatrics* 123(5): e871–77.

Kanaya, Y. et al. 2006. “Maturational Alterations of Peripheral T Cell Subsets and Cytokine Gene Expression in 22q11.2 Deletion Syndrome.” *Clinical and Experimental Immunology* 144(1): 85–93.

Kanderova, Veronika et al. 2018. “Lymphoproliferation, Immunodeficiency and Early-Onset Inflammatory Bowel Disease Associated with a Novel Mutation in Caspase 8.” *Haematologica* 103.

Kawame, Hiroshi et al. 2001. “Graves’ Disease in Patients with 22q11.2 Deletion.” *Journal of Pediatrics* 139(6): 892–95.

Kawano, Y, T Noma, and J Yata. 1994. “Regulation of Human IgG Subclass Production by Cytokines. IFN-Gamma and IL-6 Act Antagonistically in the Induction of Human IgG1 but Additively in the Induction of IgG2.” *Journal of immunology (Baltimore, Md. : 1950)* 153(11): 4948–58.

Kelley, Clair M et al. 1998. “Helios, a Novel Dimerization Partner of Ikaros Expressed in the Earliest Hematopoietic Progenitors.” *Current Biology* 8(9): 508–15.

Klein, Ludger, Bruno Kyewski, Paul M Allen, and Kristin A Hogquist. 2014. “Positive and Negative Selection of the T Cell Repertoire: What Thymocytes See (and Don’t See).” *Nature reviews - Immunology* 14(6): 377–91.

Klocperk, Adam et al. 2014. “Helios Expression in T-Regulatory Cells in Patients with Di George Syndrome.” *Journal of Clinical Immunology* 34(7): 864–70.

———. 2015. “Low Marginal Zone-like B Lymphocytes and Natural Antibodies Characterize Skewed B-Lymphocyte Subpopulations in Del22q11 DiGeorge Patients.” *Clinical Immunology* 161(2): 144–49.

———. 2018. “Follicular Helper T Cells in DiGeorge Syndrome.” *Frontiers in Immunology* 9(July): 1–9.

Kohler, Siegfried, and Andreas Thiel. 2009. “Life after the Thymus: CD31+ and CD31- Human Naive CD4+ T-Cell Subsets.” *Blood* 113(4): 769–74.

Králíčková, Pavlína et al. 2018. “CVID-Associated Tumors – Czech Nationwide Study Focused on Epidemiology, Immunology and Genetic Background in a Cohort of Patients with CVID.” *Frontiers in Immunology*.

Kruetzmann, Stephanie et al. 2003. “ Human Immunoglobulin M Memory B Cells Controlling Streptococcus Pneumoniae Infections Are Generated in the Spleen .” *The Journal of Experimental Medicine* 197(7): 939–45.

Ladi, Ena, Xinye Yin, Tatyana Chtanova, and Ellen a Robey. 2006. “Thymic Microenvironments for T Cell Differentiation and Selection.” *Nature Immunology* 7(April): 338–43.

Lahtrop, Stephanie K et al. 2012. “Peripheral Education of the Immune System by Colonic Commensal Microbiota.” *Nature* 478(7368): 250–54.

Lima, K et al. 2010. “Low Thymic Output in the 22q11.2 Deletion Syndrome Measured by CCR9+CD45RA+ T Cell Counts and T Cell Receptor Rearrangement Excision Circles.” *Clinical and experimental immunology* 161(1): 98–107.

Lischner, Harold W, and Angelo M DiGeorge. 1969. “Role of the Thymus in Humoral Immunity.” *Lancet*: 1044–49.

Lischner, Harold W, Hope H Punnett, and Angelo M DiGeorge. 1967. “Lymphocytes in Congenital Absence of the Thymus.” *Nature* 214.

Liu, Zheng, and Anne Davidson. 2011. “BAFF and Selection of Autoreactive B Cells.” *Trends in Immunology* 32(8): 388–94.

López-Granados, Eduardo et al. 2005. “A Genotype-Phenotype Correlation Study in a Group of 54 Patients with X-Linked Agammaglobulinemia.” *Journal of Allergy and Clinical Immunology* 116(3): 690–97.

Ma, Cindy S. et al. 2015. “Monogenic Mutations Differentially Affect the Quantity and Quality of T Follicular Helper Cells in Patients with Human Primary Immunodeficiencies.” *Journal of Allergy and Clinical Immunology* 136(4): 993–1006.

Ma, Jie et al. 2012. “Increased Frequency of Circulating Follicular Helper T Cells in Patients with Rheumatoid Arthritis.” *Clinical and Developmental Immunology* 2012.

Mackay, Fabienne, Pascal Schneider, Paul Rennert, and Jeffrey Browning. 2003. “BAFF AND APRIL: A Tutorial on B Cell Survival.” *Annual review of immunology* 21: 231–64.

Maglione, Paul J. et al. 2019. “BAFF-Driven B Cell Hyperplasia Underlies Lung Disease in Common Variable Immunodeficiency.” *JCI Insight* 4(5).

Manthiram, Kalpana, Qing Zhou, Ivona Aksentijevich, and Daniel L Kastner. 2017. “The Monogenic Autoinflammatory Diseases Define New Pathways in Human Innate Immunity and Inflammation.” *Nature Immunology* 18(8): 832.

Marcovecchio, Genni Enza et al. 2019. “Thymic Epithelium Abnormalities in DiGeorge and Down Syndrome Patients Contribute to Dysregulation in T Cell Development.” *Frontiers in Immunology* 10(March): 1–15.

Markert, M. Louise, Blythe H. Devlin, and Elizabeth A. McCarthy. 2010. “Thymus Transplantation.” *Clinical Immunology* 135(2): 236–46.

Markert, M L et al. 1999. “Transplantation of Thymus Tissue in Complete DiGeorge Syndrome.” *The New England Journal of Medicine* 341(16): 1180–89.

Mayumi, M et al. 1989. “Di George Syndrome with Hypogammaglobulinaemia: A Patient with Excess Suppressor T Cell Activity Treated with Fetal Thymus Transplantation.” *European Journal of Pediatrics* 148(6): 518–22.

McGhee, Sean A., Maria Garcia Lloret, and E. Richard Stiehm. 2009. “Immunologic Reconstitution in 22q Deletion (DiGeorge) Syndrome.” *Immunologic Research* 45(1): 37–45.

McLean-Tooke, A., D. Barge, G. P. Spickett, and A. R. Gennery. 2011. “Flow Cytometric Analysis of TCR Vβ Repertoire in Patients with 22q11.2 Deletion Syndrome.” *Scandinavian Journal of Immunology* 73(6): 577–85.

McLean-Tooke, Andrew, Dawn Barge, Gavin P Spickett, and Andrew R Gennery. 2008. “Immunologic Defects in 22q11.2 Deletion Syndrome.” *Journal of Allergy and Clinical Immunology* 122(2): 362–67.

Meechan, D. W. et al. 2007. “When Half Is Not Enough: Gene Expression and Dosage in the 22q11 Deletion Syndrome.” *Gene Expression* 13(6): 299–310.

Mogues, Tirsit, Junzhi Li, John Coburn, and David J. Kuter. 2005. “IgG Antibodies against Bovine Serum Albumin in Humans - Their Prevalence and Response to Exposure to Bovine Serum Albumin.” *Journal of Immunological Methods* 300(1–2): 1–11.

Morita, Rimpei et al. 2011. “Human Blood CXCR5+CD4+ T Cells Are Counterparts of T Follicular Cells and Contain Specific Subsets That Differentially Support Antibody Secretion.” *Immunity* 34(1): 108–21.

Morsheimer, Megan, Terri F. Brown Whitehorn, Jennifer Heimall, and Kathleen E. Sullivan. 2017. “The Immune Deficiency of Chromosome 22q11.2 Deletion Syndrome.” *American Journal of Medical Genetics, Part A* 173(9): 2366–72.

Müller, W et al. 1989. “The DiGeorge Sequence II. Immunologic Findings in Partial and Complete Forms of the Disorder.” *European Journal of Pediatrics* 149(2): 96–103.

Murphy, K C, L A Jones, and M J Owen. 1999. “High Rates of Schizophrenia in Adults with Velo-Cardio-Facial Syndrome.” *Archives of general psychiatry* 56(10): 940–45.

Óskarsdóttir, S., E. Holmberg, A. Fasth, and K. Strömland. 2008. “Facial Features in Children with the 22q11 Deletion Syndrome.” *Acta Paediatrica, International Journal of Paediatrics* 97(8): 1113–17.

Patel, Kiran et al. 2012. “Immunoglobulin Deficiencies: The B-Lymphocyte Side of DiGeorge Syndrome.” *The Journal of pediatrics* 161(5): 950–53.

Picard, Capucine et al. 2018. “International Union of Immunological Societies: 2017 Primary Immunodeficiency Diseases Committee Report on Inborn Errors of Immunity.” *Journal of Clinical Immunology* 38(1): 96–128.

Pignata, Claudio, Michele Fiore, et al. 1996. “Congenital Alopecia and Nail Dystrophy Associated with Severe Functional T-Cell Immunodeficiency in Two Sibs.” *American Journal of Medical Genetics* 65(2): 167–70.

Pignata, Claudio, Antonio D’Agostino, et al. 1996. “Progressive Deficiencies in Blood T Cells Associated with a 10p12-13 Interstitial Deletion.” *Clinical Immunology and Immunopathology* 80(1): 9–15.

Piliero, Lisa M et al. 2004. “T-Cell Homeostasis in Humans with Thymic Hypoplasia Due to Chromosome 22q11.2 Deletion Syndrome.” *Blood* 103(3): 1020–25.

Pillai, Shiv, and Annaiah Cariappa. 2009. “The Follicular versus Marginal Zone B Lymphocyte Cell Fate Decision.” *Nature Reviews Immunology* 9(11): 767–77.

Ponsford, Mark J et al. 2018. “Hyper IgE in the Allergy Clinic- When Is It Primary Immunodeficiency?” *Allergy*.

Ponsford, Mark J, William Rae, and Adam Klocperk. 2018. “What’s New in HIES? Recent Insights from the Interface of Primary Immune Deficiency and Atopy.” *Current Opinion in Allergy and Clinical Immunology* 18: 445–52.

Puga, Irene et al. 2012. “B–Helper Neutrophils Stimulate Immunoglobulin Diversification and Production in the Marginal Zone of the Spleen.” *Nature Immunology* 13(2): 170–80.

Rauch, A. et al. 2005. “Systematic Assessment of Atypical Deletions Reveals Genotype-Phenotype Correlation in 22q11.2.” *Journal of Medical Genetics* 42(11): 871–76.

Repetto, G. M. et al. 2009. “Clinical Features of Chromosome 22q11.2 Microdeletion Syndrome in 208 Chilean Patients.” *Clinical Genetics* 76(5): 465–70.

Revy, Patrick et al. 2000. “Activation-Induced Cytidine Deaminase (AID) Deficiency Causes the Autosomal Recessive Form of the Hyper-IgM Syndrome (HIGM2).” *Cell* 102(2): 565–75.

Roberts, Catherine, Sarah M. Ivins, Chela T. James, and Peter J. Scambler. 2005. “Retinoic Acid Down-Regulates Tbx1 Expression in Vivo and in Vitro.” *Developmental Dynamics* 232(4): 928–38.

Ryan, A K et al. 1997. “Spectrum of Clinical Features Associated with Interstitial Chromosome 22q11 Deletions : A European Collaborative Study.” *Journal of Medical Genetics* 34: 798–804.

Sebastian, M et al. 2016. “Helios Controls a Limited Subset of Regulatory T Cell Functions Mathew.” *Journal of Immunology* 196(1): 144–55.

Šedivá, Anna et al. 2005. “Early Development of Immunity in DiGeorge Syndrome.” *Medical science monitor* 11(4): CR182-7.

Shprintzen, Robert J. 2008. “Velo-Cardio-Facial Syndrome: 30 Years of Study.” *Developmental Disabilities Research Reviews* 14(1): 3–10.

De Smedt, B et al. 2003. “Pre-Academic and Early Academic Achievement in Children with Velocardiofacial Syndrome (Del22q11.2) of Borderline or Normal Intelligence.” *Genetic counseling (Geneva, Switzerland)* 14(1): 15–29.

Smulski, Cristian R., and Hermann Eibel. 2018. “BAFF and BAFF-Receptor in B Cell Selection and Survival.” *Frontiers in Immunology* 9(October): 1–10.

Solot, C B et al. 2000. “Communication Disorders in the 22q11.2 Microdeletion Syndrome.” *Journal of Communication Disorders* 33(215): 187–204.

Staple, L et al. 2005. “Allergies in Patients with Chromosome 22q11 . 2 Deletion Syndrome (DiGeorge Syndrome/Velocardiofacial Syndrome) and Patients with Chronic Granulomatous Disease.” *Pediatric Allergy and ImmunologyPediatric Allergy and Immunology* 16: 226–30.

Sullivan, K E et al. 1999. “Longitudinal Analysis of Lymphocyte Function and Numbers in the First Year of Life in Chromosome 22q11.2 Deletion Syndrome (DiGeorge Syndrome/Velocardiofacial Syndrome).” *Clinical and diagnostic laboratory immunology* 6(6): 906–11.

Sullivan, Kathleen E. et al. 1997. “Juvenile Rheumatoid Arthritis-like Polyarthritis in Chromosome 22q11.2 Deletion Syndrome (Digeorge Anomalad/Velocardiofacial Syndrome/Conotruncal Anomaly Face Syndrome).” *Arthritis & Rheumatism* 40(3): 430–36.

Szabo, Krisztina et al. 2013. “Follicular Helper T Cells May Play an Important Role in the Severity of Primary Sjögren’s Syndrome.” *Clinical Immunology* 147(2): 95–104.

Takatori, Hiroaki et al. 2015. “Helios Enhances Treg Cell Function in Cooperation with FoxP3.” *Arthritis and Rheumatology* 67(6): 1491–1502.

Tantibhaedhyangkul, Usa et al. 2009. “Role of IL-7 in the Regulation of T-Cell Homeostasis in Partial DiGeorge Syndrome.” *Journal of Allergy and Clinical Immunology* 123(4): 960-962.e2.

Tézenas Du Montcel, S et al. 1996. “Prevalence of 22q11 Microdeletion.” *Journal of medical genetics* 33(8): 719.

Thornton, Angela M. et al. 2019. “Helios+ and Helios- Treg Subpopulations Are Phenotypically and Functionally Distinct and Express Dissimilar TCR Repertoires.” *European Journal of Immunology*: 1–50.

Thornton, Angela M et al. 2010. “Expression of Helios, an Ikaros Transcription Factor Family Member, Differentiates Thymic-Derived from Peripherally Induced Foxp3+ T Regulatory Cells.” *Journal of immunology (Baltimore, Md. : 1950)* 184(7): 3433–41.

Thrasher, Adrian J., and David A. Williams. 2017. “Evolving Gene Therapy in Primary Immunodeficiency.” *Molecular Therapy* 25(5): 1132–41.

Ueno, Hideki. 2016. “T Follicular Helper Cells in Human Autoimmunity.” *Current Opinion in Immunology* 43: 24–31.

Unger, Susanne et al. 2017. “The TH1 Phenotype of Follicular Helper T Cells Indicates an IFN-γ-Associated Immune Dysregulation in Patients with CD21low Common Variable Immunodeficiency.” *Journal of Allergy and Clinical Immunology* 141(2): 730–40.

Verloes, a et al. 1998. “Juvenile Rheumatoid Arthritis and Del(22q11) Syndrome: A Non-Random Association.” *Journal of medical genetics* 35(11): 943–47.

Vetrie, David et al. 1993. “The Gene Involved in X-Linked Agammaglobulinaemia Is a Member of the Src Family of Protein-Tyrosine Kinases.” *Nature* 361: 226–33.

Warnatz, Klaus et al. 2009. “B-Cell Activating Factor Receptor Deficiency Is Associated with an Adult-Onset Antibody Deficiency Syndrome in Humans.” *Proceedings of the National Academy of Sciences of the United States of America* 106(33): 13945–50.

Weller, Sandra et al. 2012. “IgM+IgD+CD27+ B Cells Are Markedly Reduced in IRAK-4 –, MyD88- , and TIRAP- but Not UNC-93B – Deficient Patients.” *Blood* 120(25): 4992–5002.

Wood, Philip M.D. et al. 2001. “A Mutation in Bruton’s Tyrosine Kinase as a Cause of Selective Anti-Polysaccharide Antibody Deficiency.” *Journal of Pediatrics* 139(1): 148–51.

Yagi, Hisato et al. 2003. “Role of TBX1 in Human Del22q11.2 Syndrome.” *Lancet* 362: 1366–73.

Yamasaki, Sho et al. 2006. “Mechanistic Basis of Pre-T Cell Receptor-Mediated Autonomous Signaling Critical for Thymocyte Development.” *Nature Immunology* 7(1): 67–75.

Yates, Kathleen et al. 2018. “Comparative Transcriptome Analysis Reveals Distinct Genetic Modules Associated with Helios Expression in Intratumoral Regulatory T Cells.” *Proceedings of the National Academy of Sciences* 115(9): 2162–67.

Zemble, R et al. 2010. “Secondary Immunologic Consequences in Chromosome 22q11.2 Deletion Syndrome (DiGeorge Syndrome/Velocardiofacial Syndrome).” *Clinical Immunology* 136(3): 409–18.

Zhang, J et al. 2001. “Cutting Edge: A Role for B Lymphocyte Stimulator in Systemic Lupus Erythematosus.” *Journal of immunology (Baltimore, Md. : 1950)* 166(1): 6–10.

Zhang, X et al. 2015. “Circulating CXCR5+CD4+ Helper T Cells in Systemic Lupus Erythematosus Patients Share Phenotypic Properties with Germinal Center Follicular Helper T Cells and Promote Antibody Production.” *Lupus* 24(9): 909–17.

ATTACHED MANUSCRIPTS