Prague 05.09.2019

Examiner's Report on MUDr. Adam Klocperk's PhD Thesis:

Adaptivní imunita u pacientůns primárními imunodeficiencemi

Adaptive immune system in patients with primary immunodeficiencies

This study describes an investigation into the disturbances of cellular and humoral components of adaptive immune system of patients with partial DiGeorge syndrome carrying a hemizygous deletion of chromosome 22q11.2. While the advancement in understanding of genetic causes underpinning this most common microdeletion in human populations has been quite remarkable, to ascertain underlying mechanism causing combined cardiac anomalies, hypoparathyroidism, developmental delay, psychiatric issues and immune dysfunction, each accompanied with a continuos spectrum of severity, prove to be much more difficult and a very complex task.

The recognition of this syndrome in 1965 by Angelo di George was linked to the congenital absence of thymus and parathyroid glands, which, as it turned out, shared a common embryonic developmental origin from the third pahryngeal pouch which forms approximately four weeks after the conception. Since around 8-30% of children with 22q11.2del suffer from various types of autoimmune conditions, there is an urgent need to understand underlying causes of these autoimmune manifestations. Since patients with DiGeorge syndrome suffer from a limited thymic volume, the prediction is that most of autoimmune conditions are directly linked to the dysregulated T cell biology. However, as the thymic cortex and medulla regions seem intact and preserved in patients with 22q11.2del, it is not clear which processes of T cell maturation and differentaition are impacted. By the same token, little is known how B cells, whose somatic hypermutations, switching to memory phenotype, antibody production and responses to vaccine are dependent on T cell help, will be affected in these patients. The work and results of Dr. Klocperk represent important steps towards the resolution of these uncertainities as they tengibly advance this field towards deeper understanding of immunological proceses undergoing in patients with DiGeorge syndrome.

It is necessary to emphasize that papers published from these studies are at the frontier in this area of clinical research and have been published along with studies from other, world renowned laboratories, such as the lab of Kathleen Sulivan, in USA, and Correa-Rocha, in Spain. Also, they are published in internationally recognized and impacted journals, such as Frontiers in Immunology (IF=5,69), Journal of

Clinical Immunology (IF=3,9) and Clinical Immunology (IF=4,034). In this regard, the work represents an indispensable addition to scientific literature publically available on this topic worldwide.

Results are indeed very interesting. Notably, Dr. Klocperk was able to demonstrate that predictions concerning possible causes of autoimmune conditions in patients with DiGeorge syndrome are not always true and often contradict experimental expectations. For example, while the total number of T cells, as well as Tregs, are diminished in these patients, their ratio remains more or less unchanged, suggesting that Tregs are likely not the cause of autoimmunity. Similarly, deficiencies in B cell maturation and class switching are not due to the expected lower amount of circulating follicular helper T cells (cTFHs) which are rather elevated. Contrary, by considering their numbers, cTFHs could not be seemingly accounted for deficiences in the production of antibodies and the onset of autoimmunity and allergy in these patients. However, as the authors described for the very first time, changes in the expression profiles of two critical auxillary costimulatory molecules on cTFHs, PD1 and ICOS, might be important for observed immune dysfunction in patients with DiGeorge syndrome. Many other interesting results are clearly presented in the thesis and attached manuscripts which together provide a more comprehensive, and in some aspect quite unexpected view of distorted mechanisms which might be relevant to autoimmune conditions in these patients. Together, presented results pave the way for better understanding how the process of breaching tolerance can be evaluated in patients with DiGeorge syndrome in more complex and accurate way. The impact on clinical practice could be potentially far reaching as a step-by-step improvement in this proces can potentially improve patient's lives.

The thesis is well written. It is presented in a shorten version whereby it contains the abstract and Intro chapter which highlights the history, classification of PIDs, and then focuses on a full description of DiGeorge syndrome from the point of view of its history, causes, clinical phenotype, immunity and therapeutic options. The chapter "Aims of the thesis" introduces the main goals of this study followed by the list of Adam Klocperk's published works. This portfolio consists of impressive 10 publications, where Dr. Klocperk is the first author on three primary papers. Major achievements of these studies are concisely summarized in a separate chapter "Summary of published work". Analysis of results and clearly stated conclusions in the first-author publications of Dr. Klocperk attest for his significant achievement in this relatively highly competitive field of research.

While I feel that the conclusions of this study are very important and strong, there are several suggestions and questions that could be further discussed. First, I have several formal concerns:

1/ While it is obvious that the thesis is written mostly from the point of view of a clinician, it would be beneficial for the reader if the Intro chapter would also include a paragraph and figure highlighting the genetic causes and mechanism of chromosome 22q11.2 deletion. This complex process should be then linked to explain the variety of clinical phenotypes within the group of patients with DiGeorge syndrom and their heterogeneity even within families. List of genes and other cis- and trans-regulatory units and elements affected by such deletion should be highlighted to give reader a broader view of the scale of genetic burden which such deletion brings about to relevant patients.

2/There are several places in the text and even whole paragraphs without any cross-reference to published reports (for example on the page 21, 23, 28, 34) which make the relevant statements difficult to verify.

3/ Figure 2 shows the histology of the thymus from a healthy individual. Given the topic of the thesis, it would be beneficial for the reader to compare it to the image(s) of analogous internal structure of thymi from DiGeorge patients with decreased thymic volume(s).

Questions for discussion:

1/ It is not obvious how the 22q11.2 hemizygosity can have such a profound physiological impact reflected in the spectrum of phenotype, scale of severity, and heterogeneity within individuals in the affected population and even within families. What is the current mechanistic view on underlying causes of these observed accompanying conditions?

2/ The relationship between the nomenclature referred to “DiGoerge syndrome” and 22q11.2 deletion is generally somewhat confusing. While the latter relates to a chromosomal region variable in size and precise localization, the former is mostly defined by phenotype which extremely varies in these patients. Moreover, only one in approx. 500 patients with 22q11.2del manifests the full DiGeorge syndrome, while 8-30% display the the partial DiGeorge syndrome. Yet, the only diagnostic criterium for DiGeorge by ESID is the reduced number of CD3 lymphocytes, which, on the other side, also accompanies many other types of immunodefieciencies. In addition, while the deletion of TBX1 seems to be a better predictor for decreased T cell counts and overall risk of autoimmunity in DiGeorge syndrome, this criterium is, in general, not used for diagnosis. In your paper (*J. Clin. Immunol., 2014*) the vast majority of patients with DiGeorge syndrome exhibited the total number of T cells in blood still within 5th and 95th percentile of published healthy values. Thus, in this regard, it is not quite clear, how patients are selected for the studies? Are they indeed immunocompromised? Is their thymus volume assessed by X-ray image? Is there any protocol for the stratification of patients according to

some additional criteria which should indicate their dysbalanced immunity? As an internationally recognized laboratory, would you recommend different type of protocol for determining a subset of patients with DiGeorge syndrome in which the study of dysbalanced immunity would be beneficial for improving their quality of life?

3/ In your studies, as a control, you often used referential age-related values, which relate to data generated and published more than 20 years ago (*Comans-Bitter, W.M., J. Pediatr., 1997*). Given that different type of staining antibodies, protocols and FACS machines are used, it is not clear how these datasets are made comparable and how they are internally normalized. What are the bases for justification of direct comparison of FACS data from your dataset and those from referential depositions? Would not be more appropriate to include a freshly recruited healthy donor to your studies?

4. On the page 25, the author referred to a study in which the expression of Aire in DiGeorge patients was diminished, suggesting a very complex regulatory network converging on impacted function of mTECs. Given that TBX1 and FoxN1 are other two key transcription factors (TFs) regulating the development of thymus and establishment of tolerance, is anything known about the functional relationships between these three TFs during ontogenesis?

5. While the chapter "Summary of published work" clearly states achieved results, it would be beneficial, to generalized these data and explain how they contributed to the elucidation of immunological symptoms observed in DiGeorge patients, including allergy and astma (to which you refer on the page 17). In other words, which specific immune mechanisms in patients with DiGeorge are seemingly the most affected? Can these affected mechanism(s) explain the plethora of immune deficiencies and conditions observed in patients with DiGeorge syndrome. Can you present a slide which would summarize such a generalized view?

6. On page 25, the author states that, quote: "For comparison of interdepartmental differences, no thymic tissue is found by surgeon when performing corrective cardiac surgery in infants with DiGeorge syndrome at the author hospital". Can you please elaborate on this statement, as it is not clear to what specific type of interdepartmental discrepancy this refers to. It is a diagnostic or patient's stratification problem, or something else?

7. Lastly, there are several technical questions and comments to the paper published by the Dr. Klocperk in *J Clin. Immunol., 2014.* First, very interesting fact highlighted in the Fig.1 of this paper is that patient's samples, compare to those of healthy donors, lack bigger and more granular subset(s) of

cells seen above and to the right of the lymphocyte gate. Is this a general phenomenon? What specific type of cells are these? Second, in the same Fig.1A, it not clear how the positive gates for FoxP3 and CD25 markers were set up. Can you show the FMO control? Do patient's and healthy donor's sample differ in MFI of these two parameters? Third, it seems that the numerical label on y-axis of Fig.1C and Fig.3B is incorrect and should read: Tregs[x10^6/ml].

Conclusions and recommendation

I have identified both the strengths and weaknesses of the thesis, although I have concentrated mainly upon the latter as is expected in such report. I want to emphasize however, that the above listed concerns in no way diminish the high quality of work presented in this thesis and author's publications.

The thesis of Dr. Klocperk is a very important work presented in a well-written shortened format that brought further advancement in re-evaluation of underlying immune mechanisms in patients with DiGeorge syndrome. This contribution, in a long run, can improve the management of these patients with respect to their compromised immune system. In addition, up-to-date approaches to conduct clinical research, open presentation and discussion with decent analysis of obtained results as well as the ability of Dr. Klocperk to coordinate and integrate his clinical and experimental work, demonstrate that the author is fully prepared for his professional clinical carrier. His papers published in a well recognized international journal lend further support for this statatement. Given the quality of Dr. Klocperk's work, I fully recommend this thesis to be accepted as the fulfilment of the requirement for awarding PhD degree to the candidate according to the law §47 section 4.

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