Ph.D. Thesis evaluation report

Immunophenotypic and functional characteristics of lymphocytes of patients with primary Immunodeficiency

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This dissertation thesis is focused mainly on the immunophenotype of lymphocytes obtained from patients with primary immunodeficiencies (PIDs). This topic is quite important as the detailed characterization of these inborn defects is essential not only for a precise diagnostics but also for subsequent therapeutical decisions. In addition to conventional flow cytometry, multiparameter mass cytometry was used supplemented by functional tests and advanced computational analysis of the data.

In the **Introduction**, the main features of development of T cells are described, and mentioned potential steps involved in the development of of immunodeficiencies. After describing development in the thymus and thymic egress, recent thymic emigrants and following differentiation in the periphery leading to subsets of effector and memory cell is shown. Similarly, differentiation of B cells in the bone marrow and secondary lymphoid tissues is described. In the next part of the text, an overview of primary immunodeficiencies is given. It begins with severe combined immunodeficiency (SCIDs) including those with defective survival of hematopoietic cells, defective V(D)J recombination, toxic metabolite accumulation, defective pre_TCR and TCR signaling and defects of cytokine signalling. Less severe forms of combined immunodeficiencies represent atypical SCIDs and other less profound CIS based on immune dysregulation (defective T cell survival or TCR signaling, DOCK proteins deficiency, and MHC II deficiency. The Wiskott-Aldrich syndrome, ataxia telangiectasia, and DiGeorge syndrome are categorized as CIDs with associated or syndromic features. Diseases of immune dysregulation are represented by autoimmune lymphoproliferative syndrome and by IPEX, a mutation in the FOXP3. In the part dedicated to predominantly antibody deficiencies, four forms of agamaglobulinemia are described, hyper IgM syndroms, and common variable immune deficiency are mentioned together with relatively common IgA and IgG subclasses immune deficiencies which are asymptomatic or having relatively favourable clinical outcomes. Defects in innate immunity are described relatively briefly

since the dissertation is focused mainly on lymphocytes and adaptive immunity. The next part of the Introduction deals with different diagnostic approaches including T cell receptor excision circles (TRECs) and kappa-deleting recombination excision circles (KRECs), genetic testing, flow cytometry, and functional tests. Also, the mass cytometry functional immunophenotyping is described used mostly in research, so far. Finally, a brief description is given of the therapy such as hematopoietic stem cell transplantation, gene therapy, targeted therapies with small molecules or monoclonal antibodies, and immunoglobulin replacement therapy.

The specific **aims** of this thesis were to contribute to improvement in diagnostics of patients suspected of having PIDs, predominantly antibody deficiencies, and more specifically explore the role of Fas signaling in the extrafollicular v.s. germinal center decisions in ALPS/FAS patients. The final specific aim was to characterize a novel mutation in the TLR8 gene associated with autoimmunity and autoinflammation.

The laboratory **methods** are described in detail in the attached manuscripts, this thesis thus does not require to contain a comprehensive Methods part.

The **Results** are presented in five publications of which one is under revision. Mgr. Bakrdjieva is the first author in two of them (in one case, the first authorship is shared with her supervisor and one is in the review process).

The first manuscript was done in collaboration with four other laboratories of the EuroFlow consortium and this project led to the development of a standardized 8-color flow cytometric test called SCID-RTE for the identification of patients suspected of having a severe form of PID (SCID, CID). Furthermore, this test can be used also in children identified by newborn screening programs for SCID with low or absent TRECs. It showed a high sensitivity for the monitored parameters such as levels of naive CD4+ and CD8+ T cells, recent thymic emigrants (RTE), and activation status.

The next multicentric study analyzed subsets of B cells and plasma cells (PCs) in a large cohort of patients with predominantly antibody deficiencies (PADs). In all of them, decreased counts of PCSs, memory B cells (MBCs) or both expressing distinct IgA or IgG subclasses were identified. Blood PCs were found to be the most sensitive cellular

compartment whereas evaluation of blood MBCs seemed rather informative to discriminate different clinical profiles.

The following manuscript contributed to the knowledge of human B cell development by investigation of bone marrow and peripheral blood samples by 30-parameter mass cytometry to visualize the multidimensional data. This approach with a new algorithm integrating several autonomous modules showed not only expected B cell developmental stages in bone marrow and peripheral blood but added detail to the transition points and dynamics in the expression of nuclear factors, cytokine and chemokine receptors in relation to canonical B cell developmental markers and developmental branching points.

The role of non-apoptotic FAS signaling in B cell differentiation was studied in patients with ALPS with FAS mutations. In healthy subjects, FAS provided non-apoptotic signaling which contributed to germinal center (GC) versus extrafollicular (EF) developmental decisions in CD40L-activated B cells. This was regulated by the downregulation of the mTOR pathway and also by transcriptomic changes. In contrast, this mechanism is impaired in ALPS with FAS mutations leading thus to enhanced mTOR signalling, reduction of GC B cell maturation and increased EF responses. The study elucidated new role of non-apoptotic FAS signaling in B cell maturation.

The last publication supporting the thesis described a novel mutation in the TLR8 gene responsible for severe autoimmune hemolytic anemia with features of autoinflammation in identical twins. This mutation reduced the ability of TLR8 to attenuate TLR7 signaling and led to increased pro-inflammatory responses with activation of NF-κB and subsequent proinflammatory cytokine production.

I do not have any major **comments**, just a few minor ones:

In the Introduction, the part describing the development of T cells in the thymus and the periphery misses any mention of T regulatory cells. Similarly, B regulatory cells are not described.

Some of the abbreviations are not elucidated either in the text or a list of abbreviations, e.g. DOCK, LRBA, t-SNE, UMAP, …etc.

Questions:

1. Flow cytometric immunophenotyping of lymphocytes in newborns with SCID may be complicated by maternal T cell engraftment which might be relatively frequent. Is there a standardized method to detect these maternal T cells?
2. The CD73 molecule is a marker of switched memory B cells as confirmed also in this thesis. On the other hand, inhibitors of CD73 are currently tested as a potential cancer immunotherapy in pre-clinical and Phase I clinical studies. Would you expect that blocking of CD73 by a monoclonal antibody or by a small molecule inhibitor represents a potential risk factor by affecting differentiation of this B cell subset?

Conclusions:

The candidate demonstrated in-depth knowledge in primary immunodeficiencies and identified some of the problems in the field. The dissertation thesis was generally well written, fulfilled its objectives, and the results published in excellent international journals contributed significantly to current knowledge. In this respect, I am pleased to recommend Mgr. Marina Bakardjieva is to be awarded the degree of Doctor of Philosophy.

Ilja Stříž

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