Institute of Molecular Genetics AS CR, v.v.i.

Examiner's Report on MUDr. Karel Švojgr PhD Thesis:

The function of adaptor proteins in leukemogenesis.

This study describes an investigation into the characterization of the role of adaptor protein in the leukemogenesis and the mechanism underpinning their function. Presented thesis reports on results from two seemingly independent studies that overlap in terms of their unified effort to identify key molecules regulating the development and maturation of lymphocytes: adaptor proteins and phosphatase CD148. Specifically, it is focused on (i) the kinetics of expression of three important adaptor molecules NTAL, LAT and PAG during the thymic development and in main subtypes of children ALL; (ii) *in vitro* assessment of the role of NTAL in sensitizing leukemic T-lymphocytes for enhanced apoptosis after anti-TCR or glucocorticoid treatment; and (iii) the kinetics of expression of CD148 in human an mouse thymic T cell precursors.

The thesis is well written up. It consists of 8 chapters on 48 pages supplemented with the full copies of 3 original articles, on two of them dr. Švojgr as the first author. The Introduction, Background, Aim of study and a short explanation of Methods, Results and Discussion for each paper chapter help the reader to follow the logic of the candidate's argument as he constructs the rationale for the study, and shortly describes its design, procedures and the methods required for its analysis. The result sections, and in my view especially those describing of the role of NTAL in the rate of apoptosis of TCR- or glucocorticoid receptor-stimulated leukemic T cells, described in the first and second paper, are the most interesting ones to read. It clearly demonstrates the complexity and crosstalk between signalling pathways regulating the pro-apoptotic sensitivity of leukemic lymphocytes and points to the possible therapeutic utility of this observation. This experimental result is also of a general importance as it positions ERK kinase as the possible essential modulator of apoptosis of leukemic cells. Specifically, the fact that signalling from the two independent receptors, i.e. TCR and glucocorticoid receptor, converges on and regulates activation of ERK with somewhat similar effect on the survival leukemic T lymphocytes or analogous T-cell line Jurkat/NTAL^{+}, further suggests the central role of this signalling protein in a leukemic proapoptotic cell-decision process. That led the author to postulate a clinically important conclusion that the level of expression of NTAL is a suitable prediction marker for a successful initial glucocorticoid therapy, the finding which dr. Švojgr mechanistically linked to the regulation of the kinase activity of ERK.

The third parts of the thesis are also very interesting as they provide the essential information about the kinetics of expression of CD148 phosphatase during T cell development in mouse and human, the later characterized by the defendant himself. It seems that this is the very first report describing strikingly distinct expression pattern on mouse and human thymocytes subsets. Moreover, the authors in this paper also showed, that CD148, similar to CD45, can function not only as a negative, but also as a positive regulator of TCR signalling.

Karel Svojgr in these three primary publications demonstrates his skilfulness and intellectual ability to undertake and solve the relatively difficult task of elucidating the mechanism underlying the regulated apoptosis of leukemic T cells, what is always one of the most difficult aspects of biological research. Apart from these excellent results, he used appropriately chosen methodological approaches to identify critically involved signalling molecules. The fact that dr. Švojgr was able to connect the basic research with translational medicine and apply his findings to clinically relevant setting is a great achievement for PhD student.

The obvious strength of the study is the usage of clinical samples isolated from patients with various subtypes of ALL which provide a critical insight into the expression of several adaptor proteins in the lymphocytes from these patients. This is a very inspiring and, unfortunately, still rare-to-see collaborative effort which began with the discovery of several novel adaptor proteins in the lab of prof. Horejsi, which was then used and translated to clinically important study. Importantly, while we still do not fully understand the function of these molecules, the defendant was able to implement them in the regulatory process of leukemogenesis what further paves the way for elucidation of their physiological function in health and disease. It is also encouraging to see the continuous effort to expand the original "Czech-made discovery of adaptor proteins" into exploring their clinical importance. From the results presented inhere and accompanying papers it is clear that all major objectives have been largely achieved and were suitable for publications in well recognized international journals focused on immunologically, hematologically and biochemically oriented research.

However, while I feel that the thesis is very strong in its characterization of the mechanism controlling the apoptosis of leukemic T cells, there are several questions/concerns that need to be clarified:

1/ The defendant showed that anti-TCR as well as methylprednisolone treated NTAL positive cells are more sensitive to apoptosis. However, while anti-TCR stimulation clearly results in ERK phosphorylation and activation, such effect is not shown for the methylprednisolone treated leukemic cells. Rather, the author showed that U0126, the pharmacological inhibitor of ERK, reverses the sensitizing effect of methylprednisolone-treated NTAL positive Jurkat cells. What additional and more direct evidence is there to support a direct link between methylprednisolone-treated NTAL positive leukemic cells and activation of ERK?

2/ NTAL contains several tyrosine residues which when phosphorylated could be important for the nucleation and amplification of downstream signals. Have you attempted to identify the critical tyrosine residue on NTAL responsible for Grb-2 binding resulting in the proposed apoptosis-sensitizing effect?

3/ In the paper published in Experimental Hematology, on Fig. 3B an 3C, the author shows the kinetics of p-ERK after TCR stimulation measured by either FACS or Western blotting (WB). When WB is inspected visually, it seems that the increase in p-ERK is much more pronounced than just 1.5 fold as measured by FACS. Moreover, it also lasts much longer. However, no direct measurement of WB intensities is provided. How do you reconcile this apparent methodological discrepancy (FACS vs WB) in the measured activation status of ERK 5 and 30 min after TCR stimulation? Interestingly, on the same Western blot, there are apparent significant differences in the phosphorylation status of p38 between Jurkat/wt and Jurkat/NTAL^{+} cell lines. Did the author normalized these levels and established the kinetics of phosphorylation for each MAPK analysed?

4/ The author suggest that the increased sensitivity to apoptosis in $NTAL^{+}$ compared to $NTAL^{neg/low}$ leukemic cells treated with anti-TCR antibody or methylprednisolone is due to signalling converging on activation of ERK which in turn regulates the proapoptotic sensitivity of these cells. As glucocorticoid and TCR signalling pathways are seemingly independent, it would suggest that the presence of NTAL in these cells must augment ERK activation even in non-treated/non-activated cells. However, as shown in Fig. 3B and C (Experimental hematology paper), in unstimulated cells, the activation status of ERK in both $NTAL^{+}$ and $NTAL^{neg/low}$ Jurkat cells is comparable and largely undetectable. Can the author suggest other possible mechanisms of NTAL-mediated increased sensitivity of leukemic cells to apoptosis via ERK-regulated process?

5/ The very last question is whether the laboratory testing for NTAL positivity among T-ALL patients is under consideration for clinical setting in the Motol hospital? Current literature lists several other expression markers for this type of disease that could be used together with NTAL expression. Can you summarized the progress in this field in last several years and how it impacts on the stratification of ALL patients?

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 a report. I want to emphasize however, that the above listed concerns in no way diminish the high quality of work presented in the thesis.

MUDr. Karel Švojgr's thesis represents a first class work presented in a well-written standard format that brought significant advancement in the field of leukemogenesis. Multiple experimental approaches, many advanced procedures and techniques described, open presentation and discussion with decent analysis of obtained results demonstrate that the author is fully prepared for the scientific/clinical carrier he has chosen and is able to work independently. The author has already published three papers in well recognized international journals specialized in this topic in biological research.

Given the quality and the experimental richness of MUDr. Karel Švojgr 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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