Kraków, 12.02.2020

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EVALUATION

of the Doctoral Dissertation prepared by Anna Piaszyk-Borychowska, MSc,

a PhD student at the Institute of Molecular Biology and Biotechnology,

Faculty of Biology, Adam Mickiewicz University in Poznań

Title of thesis: "Genome-wide characterization of STAT1 and NFκB-mediated signal integration in vascular inflammation"

Faculty of Biochemistry,
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and Biotechnology

Department
of Medical Biotechnology

prof. Alicja Józkowicz, PhD, DSc

For a long time the studies on atherogenesis have focused on analysis of dyslipidemia, endothelial dysfunction and macrophage-mediated inflammation. Vascular smooth muscle cells (VSMCs) were given less attention even though they constitute the major cells in the media of arteries and participate in arterial wall remodeling. Currently it became clear that VSMCs play important roles in atherosclerosis throughout all stages of plaque formation, and that disease progression involves a crosstalk of VSMCs with both endothelial cells and immune cells. The underlying mechanisms of such crosstalk remain, however, poorly understood.

VSMCs in advanced lesions have been generally regarded as athero-protective and plaque-stabilizing cells whereas macrophages have been considered as being proinflammatory and athero-promoting. Now, it became clear that this model may be overly simplistic. Therefore, providing the unbiased and comprehensive data that directly compare the response of macrophages and VSCMs to the same pro-inflammatory stimuli, is a valuable step towards better understanding of cell-specific functions and interactions. Obtaining such data was the basic aim of the Doctoral Dissertation prepared by Ms. Anna Piaszyk-Borychowska.

Nearly all molecular studies of macrophages have until now mainly focused on primary cells exposed *in-vitro* to single, strongly polarizing ligands, with lipopolysaccharide (LPS), interferon gamma (IFNγ) as the most intensively investigated paradigms. Such models are, however, insufficient for understanding the real mechanisms governing plaque development, as macrophages and VSMCs *in-vivo* are exposed to a broad range of stimuli whose integration determines transcriptional and functional outputs. The Candidate, Ms. Anna Piaszyk-Borychowska took on to clarify in her doctoral thesis some aspects of these relationships. Her work,

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performed under supervision of prof. Johannes Bluyssen, is a direct continuation and logical follow-up of previous studies carried out and published by the team. Experience of the Supervisor and his support was undoubtedly important for the success of the performed research.

The main goal of the Candidate was to characterize the mechanism of priming-induced signal integration between interferon alpha (IFNα), IFNγ, and lipopolysaccharide (LPS) in VSMCs, as compared to macrophages and dendritic cells (DCs). Analyses focused both on gene upregulation and gene suppression, the latter being much less recognized in previous studies. In the Dissertation the Candidate was able to identify either the common pathways active in macrophages and VSCMs or cell-type specific gene expression patterns induced by proinflammatory factors. Moreover, she proposed a potential mechanism that might explain the observed similarity in signal integration in different cell types as well as comparable effects of IFNα or IFNγ priming on TLR4-induced activation.

In my opinion, the most interesting results obtained by the Candidate are: i) demonstration that despite functional differences between VSMCs, macrophages and DCs, stimulation with pro-inflammatory stimuli results in activation of a common signal integration mechanism in these cells; ii) strong indication that ISGF3 complex is formed in vascular cells in response to IFNα but also to IFNγ; iii) a general model of priming-induced signal integration in which IFNα- and IFNγ-activated complexes containing STAT1 are recruited to closely spaced ISRE-NFκB or GAS-NFκB binding sites, what correlates with increased histone acetylation and induction of gene transcription. Deciphering the signal integration pathway is important to understand the roles of both IFN types in vascular inflammation and atherosclerosis progression.

Conducting of the project required from the Candidate application of unbiased genome-wide analyses of gene regulation and expression profiles in the *in-vitro* cultured primary VSMCs, macrophages and DCs, in response to IFNα, IFNγ, and/or LPS. The most important and challenging part was the comprehensive and integrative bioinformatics analysis of the obtained RNA-seq or ChIP-seq data sets. The Candidate applied among others DESeq package and PANTHER resources for gene ontology (GO) analysis or IGV, pSCAN, oPPOSUM and HOMER software tools for the promoter analysis and ChiP-seq data interpretation. Help offered by the collaborators, namely Dr. Lajos Szeles and Dr. Attila Csermely (University of Debrecen) is clearly indicated in the thesis. All raw data are publicly available and accession numbers are provided. Importantly, the methods used were mutually complementing, and supported by additional analyses, such as evaluation of gene expression at mRNA level using

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quantitative RT-PCR, ChIP-PCR or co-immunoprecipitation assays followed by western blotting. Thus, research methodology was diverse and correctly chosen to achieve the aims of the project, although it did not implicate a full experimental verification of the obtained results and suggested mechanisms.

Experiments were carried out by the Candidate using primary VSMCs, macrophages and DCs isolated from the wild-type C57BL/6 mice or constitutive total knock-outs for STAT1, STAT2 and IRF9 genes. VSMCs were isolated from the aortas by enzymatic digestion. Characterization of the obtained cells was performed at mRNA level by analysis of three genes.

- It is unclear for me, however, how this type of analysis can be used to assess a homogeneity of the cell culture (as written on page 33). Were these measurements performed at single-cell level? If yes, for how many cells? Were they supported by FACS analysis of VSMC markers?

Macrophages were cultured from the bone marrow-derived monocytes, after *in-vitro* differentiation, induced by M-CSF. Cell characterization was done by FACS for a single marker (F4/80). Similarly, DCs were derived from the bone marrow monocytic cells after *in-vitro* differentiation induced by GM-CSF, and characterized using FACS and double staining for CD11c and CD11b.

- In my opinion, more in-depth characterization of the cells subjected to the genome-wide analyses would be beneficial. First, the markers chosen are correct but not sufficient to determine cell identity (especially in case of DCs). Second, all populations are known as heterogenic with cell subsets differently responding to cytokines. I would recommend more detailed characterization of cells based e.g. on multicolor flow cytometry and – depending on the results – work on the sorted cell subsets. Of course, this is a subjective opinion resulting from my personal interest in vascular cell heterogeneity.

Dissertation by Anna Piaszyk-Borychowska consists of 181 pages of text, with 48 figures or photos and 15 tables. List of references contains 294 items. The thesis is not classically composed and consists of five differently organized chapters. Such a composition is acceptable, however, it results in many repetitions in the text, both in the descriptions of methods and in the discussion sections. I suppose that classical structure of the Dissertation would be more clear.

Whole text is preceded by Contents, while ended by References, List of figures, List of tables, List of abbreviations, List of publications coauthored by the Candidate, Acknowledgments and Streszczenie (in Polish). List of abbreviations is short and indeed consists of the most important ones. Lists of figures and tables are

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well prepared and facilitate reading of the thesis. Streszczenie is correctly written, although it looks more like a direct translation from the English version than as an original Polish text. Direct comparison with English Abstract was, however, impossible as this element has not been included in the thesis.

Chapter 1 (consisting of 26 pages) is a general introduction to the dissertation subject, correctly composed and interestingly written. It starts from a general description of inflammatory diseases with focus on pathogenesis of atherosclerosis. Then the Candidate pays a close attention to characterization of macrophages and VSMCs in healthy and inflamed vessel wall, describing the mechanisms of activation, production of inflammatory mediators and role in atherosclerotic plaque progression. Much less attention is given to DCs.

The next subject of Chapter 1 is role of interferons and LPS in induction of vascular inflammation. The Candidate stresses the signal transduction pathways and describes main transcription factors (STATs, NFκB, IRFs) activated in inflamed vessel wall. The description is detailed and illustrated with good schemes, summarizing the main message. The separate subchapter describes known elements of signal integration between interferons and LPS. Then, the Candidate describes role of STAT1 and NFκB in gene repression and in cell type specific transcriptional response to proinflammatory triggers. Underlying the controversies resulting from discrepant results published by different teams serves as a good rationale for performing further studies. The last part of the Chapter 1 regards the scope of the thesis, written not as a list of aims and research questions but rather as a short summary of the subsequent chapters.

The Chapter 1 is written in a good, communicative language, easy to follow by the reader. Figure legends are understandable and detailed. Particular subchapters create a cohesive and logical content, although transition between subsequent subchapters not always is smooth (e.g. first information on LPS and TLR4). Very interesting and timely part is the discussion of lineage commitment and cellular fate decision mechanisms with the indicated role for lineage determining and signal-dependent transcription factors (LDTF and SDTF).

There are some mental shortcuts, especially in the description of atherosclerosis development. For example, listing oxidative stress, turbulent blood flow and shear-stress as injurious stimuli for endothelial cells (page 8) can be misleading. Actually, regions exposed to laminar flow are atheroresistant and shear stress is known to induce antioxidant signals and maintain the quiescent phenotype in endothelium. Similarly, endothelial cells indeed release soluble forms of adhesion

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molecules (page 8), but rather the membrane forms do orchestrate leukocyte rolling on, adhesion to and diapedesis across the endothelium, thereby inducing the local inflammation in the vessel wall. Finally, ascribing PU.1 (page 16) as a macrophage-specific transcription factor is debatable, as it can regulate gene expression in all myeloid cells and B-cells. There are also some repetitions of information with (too) frequent use of "as mentioned before" or "abovementioned", and not all abbreviations are defined upon the first appearance. There are, however, only minor editorial comments.

In terms of substance the Chapter 1 is properly written, focuses on explaining the mechanisms of biological phenomena, and confirms a good theoretical background of the Candidate. It should be appreciated that the text refers not only the newest publications but also the older ones. Generally, interpretation of the referred papers is careful and correct, and the Chapter 1 convincingly shows gaps in knowledge to justify conducting the study.

Chapter 2 (consisting of 52 pages) summarizes data that have already been published as a research paper in the Frontiers in Immunology (Piaszyk-Borychowska et al, 2019). It consists of short Introduction, Material and Methods section, Results and Discussion. Introduction describes the purpose of the research, i.e. investigation of the mechanism of priming-induced signal integration between IFNα, IFNγ and LPS, mediated by STAT1 and p65 in VSMC, macrophages and dendritic cells.

Materials and Methods used in the study are sufficiently described, allowing repetition of the experiments. All primer sequences, as well as concentrations, catalog numbers and producers of antibodies are provided, what can be helpful. I have only minor comments and question to the presented methods:

- There are some repetitions in the text, looking like "copy-paste" approach (with the same spelling errors; e.g. page 34). This should be avoided.

- According to the description (page 35), stimulation with interferons or LPS was performed in medium containing 2% FBS (DMEM for VSMCs and RPMI for DCs) or in serum-free DMEM in case of macrophages. In my opinion such a difference should be explained or at least addressed in the Discussion, when cell-type specific response is described. Presence of serum can be important, especially when stimulation with LPS is considered. There are reports showing, for example, that in the peripheral blood mononuclear cells genes which are known to be IFN inducible, were induced by LPS only in the presence of serum. It also was shown that serum LBP (LPS binding protein) plays a critical role in regulation of the IFNβ signaling pathway by LPS, implying the possible link between LBP and activation of TLR4 and IRF3.

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- It seems that the list of genes subjected to GO analysis was created with a Fold Change threshold (FC>2 or FC>3), as a sole criterion. I would prefer to filter differentially expressed genes using both a statistical significance (e.g. an FDR < 0.1) and a Fold Change threshold.

The Results are described in details and illustrated with proper, good-quality graphs. Step-be-step description is clear and convincing. Presentation of the data in the way which underlies the logical connections between subsequent stages of work should be appreciated. Analyses are complementary and mutually supporting. An elegant approach is the use of knockout mice to confirm the significance of the transcription factors studied, especially the role of STAT1. Based on the obtained results, the Candidate concludes that there is a common mechanism of priming-induced signal integration between IFNα and LPS or IFNγ and LPS. Moreover, the results suggest that such an integration involves interaction of ISGF3 and GAF with NFκB, followed by binding to the predicted regulatory sequences in the promoters of target genes. Analysis of epigenetic modifications showed that transcriptional activation indeed correlates with increased histone acetylation and PolII recruitment. It is a good example how relatively simple experiment followed by extensive analyses may provide a general information on basic biological process, not limited to the specific experimental setting. Such an approach is valuable. The results are discussed in the context of atherosclerosis, role of interferons in vascular inflammation and similarities between VSMCs and macrophages in response to proinflammatory stimuli, based on the common integrative mechanism. I have only minor comments:

- When the group of commonly expressed genes resulting from the treatment with both IFN and LPS was identified, the overlap between macrophages and DCs was stronger than between VSMCs and immune cells (page 46). Then the analysis showed (page 47) that response of VSMCs and macrophages was more directed towards IFNγ, whereas that of DCs was primarily dependent on LPS. How these two statements can be combined?

- Western blots shown on Fig. 2.12B (page 59) and Fig. 2.16B (page 64) should not be trimmed so tightly. Bigger fragments of gels should be visible.

- The discussion is cautious, indicating additional experiments that should be carried out to confirm the proposed mechanisms. However, to a significant extent it is just a summary of the results that repeats the descriptions presented already in the Result section.

Chapter 3 (consisting of 30 pages) describes similar analyses as Chapter 2, but focuses on the downregulated genes. Short Introduction leads and justifies the

hypothesis that transcriptional complexes containing STAT1 and/or NFκB can collaborate to mediate gene repression using a similar mechanism as signal integration-dependent gene upregulation, with recruitment of HDACs or with removal of transcriptional co-activators. The obtained results allowed to identify two groups of genes. The suggested mechanism in group I relies on possible recruitment of STAT1 to GAS motifs in response to IFNγ and LPS that results in masking of the closely located NFκB binding site. In group II STAT1 and p65 co-bind to GAS and NFκB binding motifs, whereas gene repression might result from the PolII pausing. This supposition requires, however, experimental verification. The discussion of this part is very interesting but highly speculative. It should be stressed that the Candidate is aware of limitations and does not draw too far-reaching conclusions.

- I have some doubts whether collecting data on down-regulated genes in the separate chapter was reasonable, as they could be discussed together with the up-regulated genes, described in Chapter 2. This would allow to avoid many repetitions in description of methods and would support the analysis of the up-regulated genes, with more detailed characterization of the data sets, especially regarding the level of basal gene expression.

Chapter 4 (consisting of 28 pages) focuses on the role of STAT1 as a mediator of cell type specific gene expression. The short introduction is finished with important question: what is the mechanism of IFNγ-dependent gene expression in VSMCs as compared to macrophages.

- In the Material and Methods one paragraph is not clear for me (page 115). The Candidate writes that conditions of cell isolation and culture are available in Chapter 2. Then she shortly summarizes that VSMCs, macrophages and DCs were all cultured in DMEM supplemented with 2% FBS, differently than described in Chapter 2 (page 35). What was the cell culture protocol for each cell type? This might affect data interpretation.

The working hypothesis of Chapter 4 proposes the role of unrecognized LDFTs, specific for macrophages or VSMCs, which could initiate the chromatin relaxation to make it accessible for STAT1 recruitment. RNA-seq followed by the genome-wide *in-silico* promoter analysis allowed to identify a short list of potential macrophage-specific and VSMC-specific LDFTs. Importantly, *in-silico* analysis which predicted the involvement of STAT1 as a SDFT was confirmed by ChIP-seq. Experiments described in Chapter 4 indicate the hierarchical involvement of PU.1 (LDFT) and STAT1 (SDTF) in macrophage-specific gene expression induced by IFNγ. Similar mechanism, although with different LSDF(s) can be proposed for VSMCs. The Candidate clearly indicates

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which elements have been confirmed and which still require a direct experimental verification. In my opinion such inference indicates the Candidate's maturity.

Chapter 5 (consisting of 9 pages) is a general discussion of the most important results described in chapters 2-4. The Discussion is short and focuses mainly on summing up the obtained data, again, with several repetitions of earlier statements provided in the Results sections and previous discussions. Nevertheless, Chapter 5 provides a lot of interesting information, although some subjects are not strictly associated with the performed research. The valuable element of Chapter 5 is a proposal of follow-up experiments designed to experimentally verify some of the suppositions derived from the performed analyses. Two last subchapters describing the potential role of STAT1-dependent mechanism in diagnostics and in targeted therapies seem to be too speculative for me. On the other hand, the element which has not been addressed in the Discussion is functional heterogeneity of VSMCs and macrophages in different vascular beds and within the atherosclerotic plaque.

In respect of editorial work the Dissertation is prepared correctly, containing only a few spelling errors. Paper is written in English, in a communicative way. Most paragraphs are coherent and well-formed, with occasional repetitions, mainly in the Material and methods or Discussion sections. This is, however, a consequence of the structure of the Thesis and does not affect the substance.

To sum-up, research described by the Candidate is a valuable study, based on comprehensive, manifold and unbiased analyses. The obtained results improve our understanding of inflammatory response in the vessel wall. They provide also a new perspective on signal transduction pathways induced by IFNα and INFγ, as well as on signal integration mechanisms.

In my opinion, the Candidate, Ms. Anna Piaszyk-Borychowska, MSc, has achieved the aims of the study and her Dissertation meets all criteria of doctoral thesis. Therefore I recommend the Dissertation for acceptance. Due to the quality of analyzes and the ability to integrate data in search of answers to important biological questions, I propose to distinguish the Dissertation with a proper award.

Yours sincerely

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