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Assesment of the PhD thesis
of Anna Piaszyk-Borychowska, MSc
"Genome-wide characterization of STAT1 and NFκB-mediated signal integration in vascular inflammation"

Cardiovascular diseases are among the main causes of mortality worldwide, as emphasized by the Author in the Introduction to Her doctoral dissertation. The deaths due to cardiovascular diseases outweigh those caused by cancer. The consequence of this fact is a considerable interest of researchers in the causes and progression of atherosclerosis and also in finding new biomarkers of these diseases.

The Author describes in the Introduction (Chapter 1) most recent developments in this fields focusing on the role of blood vessel inflammation in atherosclerosis. She briefly characterizes this self-perpetuating process encompassing local accumulation of immune cells at an injured site of a vessel, the following production of pro-inflammatory cytokines, more cell recruitment and their transformation into foam cells. The effectors, or rather - as indicated by the results of the PhD thesis - important active players in atherosclerosis progression - are smooth muscle cells building the vessels' walls. The muscle cells respond to local inflammation by changes in the expression of specific proteins, such as contractile proteins but also pro-inflammatory cytokines. Therefore, next the Author characterizes the mechanisms regulating gene expression in cells activated by two well established pro-inflammatory mediators, i.e., interferons (IFNs) and bacterial lipopolysacharide (LPS). She emphasizes a crucial role for these responses of a synergy of transcription factors activated down-stream of the IFN I/II and LPS receptors, such as STAT, especially STAT1, IRFs and NFκB. It is worth mentioning that LPS-induced inflammation is now considered as a cause of several diseases in addition to atherosclerosis, type 2 diabetes being the most prominent example. For this reason, the results of the study undertaken by the PhD candidate are important in a broader context than cardiovascular diseases alone.

The Introduction is transparent and synthetic. The Author focuses the reader's attention in a clear and comprehensive manner on the changes in gene expression in vascular smooth

muscle cells exposed to pro-inflammatory stimuli which became her research subject. A praiseworthy feature of the Introduction are its well-designed diagrams. The ability of the Author to clearly present complex issues of gene transcription regulation, noticeable throughout the whole text, deserves recognition.

A bit surprising is the brief specification of the scope of the thesis which replaces the traditionally listed aims of the studies. In my opinion, the Author's aims of study can be summarized as: (1) an analysis of gene expression changes in the pro-inflammatory response of vascular smooth muscle cells stimulated with IFNα or IFNγ and LPS and their comparison with the gene expression in immune cells – macrophages and dendritic cells. The ultimate goal was to reveal how the signals generated integrate to augment the response in comparison to either stimulus acting separately; (2) an analysis of genes down-regulated in vascular smooth muscle cells exposed to IFNγ and LPS; (3) specification of the role of STAT1 and its collaboration with NFκB in cell-specific regulation of gene expression triggered by IFNγ. These are timely and interesting topics, the research tasks are complex and ambitious and the scope of the studies is broad.

The chapter outlining the research objectives of an unusual form is followed by also unusually built three chapters describing specific sets of experiments; each chapter with its own introduction, a description of methods used, results of studies and their discussion. The information contained in the introductions to these chapters (2-4) partly repeat the information from the general introduction to the dissertation. Even more substantial overlapping concerns the descriptions of the methods of chapters 2 and 3. Was there any particular reason for such an organization of the dissertation?

The Author conducted Her research on primary aortic smooth muscle cells, macrophages and dendritic cells differentiated from bone marrow cells, as all these three types of cells contribute to atherosclerosis. She worked on cells isolated from wild type mice and STAT1, STAT2 and IRF9 knockouts. The cell transcriptome was analyzed by RNA-sequencing (RNA-seq) while chromatin immunoprecipitation and sequencing (ChIP-seq) was used to identify the DNA sequences bound by STAT1 and the p65 subunit of NFκB. Those two demanding, genome-wide approaches were followed by elaborate bioinformatic analyses, validation/generation of selected data by ChIP-PCR, and complemented by RT-PCR and co-immunoprecipitation studies. I would like to stressed here that Ms. Piaszyk-Borychowska has mastered a wide range of modern molecular biology techniques and uses them with success.

Using this elaborate approach the Author undertook several ambitious tasks to get a detailed view of the regulation of gene transcription relevant to atherosclerosis. She obtained

the most comprehensive results when examining the cooperation of STAT1 and NFκB in the activation of gene transcription (Chapter 2). Thus, RNA-seq analysis yielded a list of over 500 genes up-regulated in vascular smooth muscle cells and macrophages and dendritic cells upon a joint stimulation with IFNα and LPS or IFNγ and LPS. Further, the ChiIP-seq analysis revealed the STAT1 and p65-binding sites in the vascular smooth muscle cells which were subsequently identified as the GAS, ISRE and NFκB motifs. Next, the Author compared the data on the transcription factor-binding sites with the list of the genes up-regulated in cells stimulated with a combination of IFNα or IFNγ and LPS (Fig. 2.11). This analysis revealed genes having single GAS, ISRE or NFκB binging sites or a combination of two or three different binding sites allowing co-operation of the corresponding transcription factors in the activation of gene expression upon a joint stimulation of vascular smooth muscle cells with IFNs and LPS.

Key to the understanding of the STAT1 and NFκB synergy was the discovery of a close proximity of the STAT1- and p65-binding sites in the cognate gene promoters. The obtained data allowed the Author to propose a model of the STAT1-NFκB co-operation in enhancing the transcriptional activity of genes in vascular smooth muscle cells as a result of increased histone acetylation. They also indicate a partially overlapping ability of IFNα and IFNγ to potentiate the LPS-induced responses in these cells. Overall, the experiments performed in this part of the dissertation are well designed, well described and insightfully discussed. Most of these results were published in Frontiers of Immunology in 2019.

Having praised the PhD candidate for her work I would like to discuss an issue concerning the conditions of cell stimulation. All experiments were performed according to a common scheme which included 8 hours of IFNα or IFNγ stimulation, 4 hours of LPS stimulation or 4 hours of IFN pre-stimulation followed by 4 hours of joint application of IFNs and LPS (according to Fig. 2.2, the description of the procedure found on page 35 is misleading). However, vascular smooth muscle cells and dendritic cells were starved prior to stimulation for 24 hours, and the text suggests that they were also stimulated in the presence of 2% FBS while macrophages - in a specially designed serum-free medium from Gibco (Materials and Methods in Chapter 2, page 35). A lack of an LPS-binding protein or albumin in the serum-free medium or their concentrations different than those supplied with 2% FBS could affect the magnitude of the activation of TLR4 by LPS in macrophages. Could this fact explain the lower responsiveness of macrophages than dendritic cells to LPS stimulation, noticed by the Author, and seen in Fig. 2.4A (right panel) and in a group of selected genes expressed in all three cell types tested and shown in Fig. 2.5A, B? As a consequence of this

apparent lower gene expression triggered by LPS, the signal integration (hence the enhancement of gene transcription) for IFNγ + LPS or IFNα + LPS was higher in macrophages than in dendritic cells. What is the Author's opinion on this subject? What was the reason for the choice of such experimental conditions?

A separate set of analyses aimed at revealing genes with down-regulated transcription in the response of vascular smooth muscle cells to IFNγ and LPS (Chapter 3). The obtained results indicate that in the down-regulation of gene transcription STAT1 dominates over NFκB. I would like to underscore that the negative regulation of pro-inflammatory responses is of utmost importance in the termination of the response of immune cells to LPS. Disturbances in these mechanisms can lead to sepsis and also to chronic low-grade endotoxemia underlying the development of atherogenesis. I wonder whether the STAT1-mediated down-regulation of gene transcription can be important also in those cells?

Finally, the Author attempted to reveal the mechanism controlling the cell-type-specific expression of genes triggered by IFNγ aiming to characterize so-called lineage-dependent transcription factors (LDTFs; Chapter 4). She compared the results of RNA-seq analyses on stimulated smooth muscle cells and macrophages with those performed in resting cells and combined them with the results of ChIP-seq studies on STAT1 binding. Eventually, She pinpointed genes that bind the LDTF called PU.1 in unstimulated macrophages which likely induces chromatin opening for subsequent STAT1 binding upon cell stimulation with IFNγ. The transcription factor(s) which could play an analogous role in smooth muscle cells remain(s), however, unidentified. Finally, She proposes that the cell-type-specific expression of genes triggered by IFNγ is likely to be determined by a combination of the activity of LDTFs and cell-specific epigenetic modifications. This chapter has a valuable discussion, including that on the function of some proteins encoded by genes activated in an IFNγ-dependent manner in vascular smooth muscle cells or macrophages.

Returning to the conditions of cell stimulation: the description in Materials and Methods to Chapter 4 says that all cells were starved and stimulated in the presence of 2% FBS (page 115). This would be an acceptable modification of the protocol if not for the fact that RNA-seq results of this series of experiments are depicted in a diagram on page 117, which is exactly the same as the diagram on page 46 in Chapter 2, Fig. 2.4A, middle panel. Fig. 2.4 was obtained as a result of experiments mentioned earlier, in which macrophages seemed to be stimulated in the special serum-free Gibco medium. Could the Author explain this inconsistency? It should be also noted that the Chip-seq analysis of STAT1-binding in macrophages was based on an external dataset concerning macrophages stimulated with IFNγ

for 1.5 hour instead of the 8 hours used in other experiments (Figs 4.4 and 4.5). This is also a good opportunity to ask: Why did the Author decide to stimulate cells for 4-8 hours?

The results of all studies are discussed shortly in Chapter 5 fulfilling the function of a final Discussion. This last chapter of the dissertation addresses with caution the possibility of using results of high-throughput analyses, such as those performed by the Author, in the identification of biomarkers of cardiovascular diseases. The whole dissertation refers to a long list of literature.

The comprehensive approach to the problem of gene transcription regulation in vascular smooth muscle cells stimulated with IFNα or IFNγ and LPS undertaken by the PhD candidate is impressive, but not without minor imperfections (some mentioned earlier). All the RNA-seq libraries (18 datasets) were prepared from at least three replicates but ChIP-seq libraries and ChIP-PCR analyses – in duplicates, which excluded calculation of statistical significance of differences but is fairly common also in good publications using similar methods. On the other hand, the RT-PCR analyses were performed in triplicates and analyzed with Anova (page 38) but no results of such analyses are shown in Figs. 2.15 and 3.8. The same applies to immunoblotting analysis of phosphorylation of STAT1 and STAT2 (Fig. 2.12B). Another omission is found in the presentation of the co-immunoprecipitation of IRF9 with STATs, where only immunoblots for STAT1 and STAT2 are shown but strangely enough no IRF9 is seen and no densitometric analysis of the results is shown (Fig. 2.14C). The same concerns co-immunoprecipitation of IRF1 and STAT1 (Fig. 2.16B). Apparently, the co-immunoprecipitation studies are rather preliminary, and since they constitute a minor element of the dissertation, they could be omitted. In Polish abstract vascular smooth muscle cells are mistakenly called komórki śródbłonka instead of komórki mięśni gładkich naczyń krwionośnych.

In conclusion, in my opinion the PhD thesis of Anna Piaszyk-Borychowska, MSc, contains original research discoveries broadening substantially our knowledge on the network of molecular mechanisms governing cellular responses to pro-inflammatory stimuli. The few critical comments included in this review do not diminish the significant value of the dissertation and should be often viewed as a discussion inspired by the Author's results. The PhD candidate has mastered the relevant research field, efficiently uses difficult state-of-the-art methods and correctly draws conclusions. The dissertation fulfills all the requirements for PhD thesis. Anna Piaszyk-Borychowska is worthy, without any doubt, of the degree of Doctor (PhD) and this is my recommendation to the Scientific Council of the Faculty of Biology of the Adam Mickiewicz University in Poznan. Taking into account the broad scope of her

studies and the value of obtained results I consider the dissertation worthy to be honored with a suitable distinction.

Rozprawa doktorska spełnia wszystkie warunki określone w art. 13 Ustawy z dnia 14 marca 2003 r. „O stopniach naukowych i tytule naukowym oraz o stopniach i tytule w zakresie sztuki" z późniejszymi zmianami (Dz. U. z 2016 r. poz. 882) stawiane rozprawom doktorskim. Wobec powyższego wnoszę do Rady Naukowej Wydziału Biologii Uniwersytetu Adama Mickiewicza w Poznaniu o dopuszczenie mgr Anny Piaszyk-Borychowskiej do dalszych etapów przewodu doktorskiego. Jednocześnie mając na względzie szeroki zakres badań i dużą wartość naukową osiągniętych wyników składam wniosek o wyróżnienie rozprawy.