Immune system regulation affected by murine experimental model of
Bronchopulmonary Dysplasia
-Genomic and epigenetic findings

Short running title; Immune system regulation and experimental BPD

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29 **Abstract:**

30 *Background:* Bronchopulmonary dysplasia (BPD) is a common cause of abrupted
31 lung development after preterm birth. BPD may lead to increased re-hospitalization,
32 more severe and frequent respiratory infections and life long reduced lung function.
33 The gene regulation in lungs with BPD is complex with various genetic and
34 epigenetic factors involved.

35 *Objectives:* The aim of the study was to examine the regulatory relation between gene
36 expression and the epigenome (DNA methylation) relevant for the immune system
37 after hyperoxia followed by a recovery period in air using a mouse model of BPD.

38 *Methods:* Newborn mice pups were subjected to immediate hyperoxic condition from
39 birth and kept at 85% O₂ levels for 14 days followed by a 14 days period in room air.
40 Next, mice lung tissue was used for RNA and DNA extraction with subsequent
41 microarray-based assessment of lung transcriptome and supplementary methylome
42 analysis.

43 *Results:* The immune system-related transcriptome regulation was affected in mouse
44 lungs after hyperoxia. A high proportion of genes relevant in the immune system
45 exhibited significant expression alterations, e.g. B cell-specific genes central to the
46 Cytokine-cytokine receptor interaction, the PI3K-AKT and the B cell receptor
47 signaling pathways. The findings were accompanied by significant DNA hyper-
48 methylation observed in the PI3K-AKT pathway and immune system relevant genes.

49 *Conclusions:* Oxygen damage could be partly responsible for the increased
50 susceptibility and abnormal response to respiratory viruses and infections seen in
51 premature babies with BPD through dysregulated genes.

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Introduction:

Bronchopulmonary dysplasia (BPD) is one of the most common and dreaded complications of prematurity as the condition may lead to morbidity and mortality in the newborn period and chronic lung disease through childhood and into adult life ¹. BPD is a risk factor for re-hospitalization during the first year of life due to respiratory infections ². The clinical consequences of chronic lung disease are also carried on into childhood with more frequent and severe respiratory tract infections ³, altered pulmonary blood flow during respiratory infections and reduced lung capacity during viral infections, amongst other symptoms ^{3,4}. Survivors of premature birth also have persisting decreased lung function parameters ⁵.

The pathomechanism of BPD is complex and largely unknown. The most important factors contributing to development of this condition include lung damage resulting from mechanical ventilation that may be necessary in premature babies, excessive oxygen therapy, as well as respiratory tract infections seen in parallel with immaturity of the immune system⁶. With improved neonatal care and techniques, a “new” BPD has emerged, with less intense inflammation and remodelling but still with problematic short- and long-term consequences ⁷. However, genetic and epigenetic factors regulating the process after disrupted lung development are also of great importance ^{8 9}.

Recently, several authors suggested a more general influence of immune system in the pathology of chronic lung diseases, such as asthma and COPD (chronic obstructive pulmonary disease), and pointed at the pivotal role of genomic pathways regulating the function of B cells ¹⁰. Inflammation as a contributing factor to the development of BPD is known and previously studied ¹¹, however there is little

known about how the lungs functions after BPD has developed and the lungs are challenged by respiratory viruses/pathogens.

Previously, we showed that prolonged hyperoxia increases DNA methylation and, subsequently, alters the expression of genes involved in the TGF-beta pathway that is crucial after disrupted lung development⁹. In the present paper we aimed to examine the mRNA expression and epigenetic effects of hyperoxia in a murine BPD model in the context of immune system regulation after BPD is de facto, and acute inflammation after hyperoxia has diminished. This is to our best knowledge the first study of its kind.

Materials and methods:

All animal experiments were approved by the Norwegian board of animal research welfare (NARA 50/13-5458). The mortality was less than 4%.

We have previously published a murine hyperoxia study of BPD¹². Briefly, litters of (C57Bl/6Tac) pups at P0 were treated with hyperoxia (85% O₂) or normoxia (21% O₂) for 14 days. The pups had free access to mothers and food/water and were kept under standard conditions. The mothers were switched in/out of hyperoxia every 24 hours. From a total of 100 pups, $n=36$ (equally distributed from the treatment groups) were prepared for histological examination (to confirm the experimental BPD) at the time of euthanasia¹³. We used morphometric assessment and determined Mean Linear Intercepts¹².

Microarray examination was performed on the remaining subjects, with $n=33$ treated with hyperoxia and $n=31$ with normoxia. All animals were sacrificed on day 28 and lung tissue was harvested.

The DNA methylation study contained 12 samples in each group, from the same animals as in the transcriptome study as the lungs were divided in 4 parts immediately after removal and snap frozen in liquid nitrogen. RNA was isolated from lung tissue using RNeasy Micro Kit (QIAGEN, Germany). Genomic DNA was isolated from tissue samples using a MasterPure DNA Purification Kit (Epicentre, Illumina, USA).

Lung transcriptome assessment:

Gene expression was analysed by SurePrint G3 Mouse Gene Expression 8x60K Microarray (Agilent)⁹. Microarray results were validated by qRT-PCR method (9

genes) using TaqMan probes^{9,12} with each reaction carried out in duplicates (numerical data published in ¹²).

Gene expression measurements were normalized and analyzed with a moderated t-test on single genes between the groups using the Limma R package¹⁴. The Benjamini-Hochbergs procedure was applied to correct for multiple testing, and genes with adjusted p-values less than 0.05 were declared statistically significant.

Single genes were studied using Gene Cards (<http://www.genecards.org/>), HUGO Gene Nomenclature Committee (<http://www.genenames.org/>), Ensembl (http://www.ensembl.org/Mus_musculus/), EMBL-EBI (<http://www.ebi.ac.uk/>), The UniProt Knowledgebase (UniProtKB) <http://www.uniprot.org/>, Mouse genome informatics <http://www.informatics.jax.org/> and NCBI gene (<http://www.ncbi.nlm.nih.gov/gene/>) for descriptions, names, functions and locations of the genes. Gene sets were analyzed through DAVID Bioinformatics Resources (<https://david.ncifcrf.gov/tools.jsp>).

Complementary methylation analysis:

The protocol for DNA methylation assessment has been described previously and the method published ⁹. Briefly, genomic DNA was extracted from snap frozen lung tissue. A total of 5 µg of purified genomic DNA was then sonicated to obtain DNA fragments ranging between 200-1000 base pairs in size. Each sample was adjusted to 250 µl with phosphate-buffered saline. Next, 200 µl was used for immunoprecipitation of methylated fragments and the remaining 50 µl as a genomic DNA reference. The genomic material was hybridized to Mouse CpG Island microarrays enabling assessment of 88,737 probes representing specific genome methylation sites in, or within 95 base pairs from 15,342 CpG islands.

We used the Genomic Workbench software (Agilent) for analysis of raw methylation data. For each microarray probe we calculated the combined Z-score value, which reflects the methylation status of a corresponding DNA fragment. A strong positive value means a probe is methylated whereas a strong negative value corresponds with non-methylation. In the statistical analysis, T-tests with Bonferroni-correction for multiple comparisons were applied to detect significant differences in the mean combined Z-scores calculated for each microarray probe between the study groups. Next, DAVID software was used to convert the list of statistically significant microarray probes to a list of genes of interest. Finally, we focused on the methylation status of genes involved in immune system related to genomic pathways that were identified in the genome expression experiment.

Results:

The results are from a whole transcriptome study that included 49 animals treated with hyperoxia and 51 animals with normoxia (control group), the method, histology and study design was published in 2018 ^{12,9} (figure 1). A heat map and hierarchical cluster analysis is added as a supplement (figure 3).

Genome expression

From this whole transcriptome microarray study from lungs from mice with oxygen induced experimental BPD $n=311$ genes were significantly differentially expressed between the normoxia and hyperoxia groups. No significant differences were seen regarding gender. From the total of 311 genes there were $n=69$ genes related to the immune system and immune cells which are presented in this paper (table 1) and have not been previously published.

Out of these 69 genes related to immunology, $n=29$ were up-regulated and $n=40$ down-regulated in the hyperoxia group compared to the normoxia group. The 69 genes were subdivided by described function belonging to the innate immune system, $n=33$ or the adaptive immune system $n=36$.

The genes were further sub-classified to; surface barriers, cytokines/excreted molecules, Natural Killer Cells, B cells (and antibodies), T-cells, CD4s (cluster of differentiation), leukocyte activities/tissue inflammation or belonging to the immunoglobulin superfamily (table 1)

Pathway analysis:

The gene list was analysed in the KEGG (Kyoto Encyclopedia of Genes and Genomes) DAVID bioinformatics database. Significant enrichment was observed for

the PI3K-Akt signaling pathway, B cell receptor signaling pathway, and the Cytokine-cytokine receptor interaction pathway.

Importantly, some of the above-listed genes are expressed almost exclusively in B cells, namely the *CD19* gene (specific receptor of the B cells), *Cxcr5*, which is a chemokine receptor, the *Blnk* gene (encodes B cell linker protein) and *Cd79b* (with *Blnk* important elements of the B cell receptor signaling pathway. The four mentioned genes were all down-regulated in lung tissue from mice treated with hyperoxia.

DNA methylation study

The analysis revealed that 58 out of 417 (14%) of genes from the PI3K-AKT pathway were hypermethylated in the hyperoxia group (table 2). Pivotal elements of the pathway (i.e. *AKT*, *PI3K* and *PTEN*) were hypermethylated (figure 2).

Discussion:

In this study, we looked at gene expression (mRNA) and epigenetic changes (DNA methylation) regarding the immune system in newborn mice lung tissue homogenate to examine the effect of 14 days of neonatal hyperoxia treatment with a 14 days recovery period. We found a high proportion of significantly differentially expressed genes related to the immune system. Pathway analysis revealed that hyperoxia induced several genomic pathways relevant to the immune system especially the PI3K-AKT pathway. When superimposed, the differential regulation in early, middle and late stages of the pathway, affected growth factors, colony stimulation factor receptors, extracellular matrix receptors and cytokines coincided in our gene lists from RNA and DNA examination (tables 1 and 2, figure 2). This gives a novel view of how the whole lung shows altered gene regulation at mRNA and DNA methylation levels after experimental BPD.

The PI3K-AKT pathway

The PI3K-AKT pathway is relevant for the immune system and has been described to regulate key effects in immune response ¹⁵. This is a large pathway regulating cell cycle and is directly related to cellular quiescence, proliferation, cancer, longevity and immune system function. The pathway also controls key aspects of B cell differentiation. Interestingly, pivotal elements of the pathway (such as *AKT*, *PI3K* and *PTEN*) were hypermethylated in our findings. This suggests decreased activity of the above-mentioned genes in the hyperoxia group. Importantly, the results from the methylation experiment complemented those from the genome expression experiment with regard to “triggers” of the PI3K-AKT pathway namely *CD19* and *Cxcr5* that were significantly down-regulated in the lungs of the animals in the

hyperoxia group. *Cxcr5* has been shown to affect T cell regulation¹⁶ and other immune system functions via PI3K-AKT signaling. The gene promotor of PTEN is hypermethylated in our findings, and is also involved in this process¹⁷.

Our findings are in line with the results from a study on rats exposed to neonatal hyperoxia¹⁸ where genes and enriched pathways like Cytokine-cytokine receptor interaction and cell cycle were involved as were genes from our study. The time-point for the study was after 14 days suggesting a sustained effect as our results coincided even after 14 days recovery following the initial 14 days of hyperoxic treatment.

The regulation of genes relevant to the innate immune system were affected, in $n = 33$ gene transcripts. In our material the genes *Asb11*, *Ccl17* and *Il12b* code for cytokines and were differentially regulated after hyperoxia treatment in our material. Innate immune system factors like cytokines are seen to drive immune cell migration during disease through chemokine signaling via PI3K-AKT¹⁹. The innate immune system is important in babies and children who have not yet fully developed adaptive immune systems.

Our findings suggest that prolonged hyperoxia results in a broad and multilevel repression of the PI3K-AKT pathway. We were not able to demonstrate synchronic alterations of expression and methylation of all crucial genes of the pathway, but this would probably require repeated experiments due to the sequential character of the hyperoxia-related biological processes. It should also be noted that persisting (as observed in our study) hyperoxia-driven DNA hypermethylation could probably be simultaneously counter-balanced by other epigenetic, proteomic and/or methylomic mechanisms, dynamically modulating genome expression in order to maintain cellular homeostasis.

B and T cell regulation:

In our material we found nine genes relevant for B-cells and antibodies. Four of these genes are expressed almost exclusively in B cells. This is the case for the abovementioned *CD19* (specific receptor of B cells) and for *Cxcr5*, which is a chemokine receptor that allows the B cells to respond to *Cxcl13* and to traffic to various body compartments, including to the pleural cavity^{20 21}.

Similarly, the *Blmk* (encodes B cell linker protein) and *Cd79b* are important elements of B cell receptor signaling pathway. These were down-regulated in mice from the hyperoxia group and may suggest the presence of decreased number and/or activity of B cells in lungs after hyperoxia treatment.

Adaptive immunity against viral infections is largely based on humoral immunity by activation of B cells and antibody secretion for neutralizing viruses to prevent further infection. Viruses seem to be more aggressive in human lungs with BPD^{2,22}, and Respiratory Syncytial virus (RSV) frequently the worst culprit.

The *Ifi2712a* gene is significantly down-regulated in our findings, and has been shown to strongly up-regulate after influenza A infections in mice.²³ It is already seen that hyperoxia treatment alone altered host response to influenza A in mice²⁴. A sustained down-regulation of a gene that should up-regulate in response to Influenza-virus infection could cause increased pathology during infection.

Hyperoxia and gene regulation

Oxygen treatment after premature birth is by itself pro-inflammatory and may illicit a negative process within the lung resulting in inflammation²⁵. By giving supplemental oxygen in the neonatal intensive care unit (NICU) this pro-

inflammatory process is perpetuated. In respiratory infections the redox status is changed even before supplemental oxygen is given and a normally developed lung might clear a viral infection with less distress and damage²⁶ than does a lung with abnormal development and disrupted gene regulation. Oxygen treatment alone seems to trigger lasting dysregulation of gene expression.

We aimed to replicate this with 14 days of hyperoxia treatment in newborn mice, and then give a 14-day recovery time to examine the lasting changes in gene expression after hyperoxia as it could be seen in a clinical situation. By not adding other factors we can see how oxygen alone affects the gene expression and gene regulation.

However, in a study in lung tissue from mechanically ventilated newborn rats with 60% oxygen treatment and LPS-injections, the authors found differentially regulated genes from the immune system, inflammatory response and hematopoiesis, similar to our findings, but after only 6 hours of treatment with oxygen²⁷.

Our findings suggest that there is a sustained effect on the gene regulation regarding the immune system in lung tissue from newborn mice subjected to hyperoxic treatment. The hyperoxia was delivered in the saccular phase of lung development in mice inducing disrupted lung development to yield experimental BPD. The differences in gene regulation in the immune system after hyperoxic treatment of the lungs might be a cause of increased severity and different response to pathogens in the lung on the whole during respiratory tract infections in persons with BPD. Our findings may give rise to targets for further research on prevention and treatment regarding the immune response in lungs with BPD.

Future studies may look further into the single genes and pathways elucidated in our study to further unravel the disrupted gene regulation left by hyperoxic treatment during lung development and to search for clues to why lung tissue displays a disordered immune system function.

Limitations:

The BPD in this model is created solely with hyperoxia causing abrupted alveolar development in mice lungs resembling that of premature human babies, but does not completely embrace the multifactorial situation that causes BPD to develop in humans. However; in the search for describing the details of the pathomechanisms involved, specifically in this paper examining the gene regulation as BPD is “settled” in the lung tissue, this could also be an advantage as confounding additional interventions/factors can be reduced²⁸.

We have not analyzed gene expression at individual cell type level and this leaves limitations regarding the interpretation of function. The PI3-AKT pathway is a large pathway and the DNA methylation changes may also reflect other differentially regulated functions in the lungs.

Mice and humans do not always share pathogens and even when they do there are many differences in both adaptive and innate immunity between humans and mice.

Mice have simpler airway anatomy (fewer bronchioles, the airway branching less complex and larger airway lumen). The immune system in adult mice shows a different ratio between neutrophils and lymphocytes compared to humans.

Also, mice have more bronchus-associated lymphoid tissue²⁹.

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Figure 1 Study design. Each lung was divided into 4 parts allowing both mRNA and DNA examination from the same animal.

Figure 2 A simplified PI3K-AKT pathway with genes either with decreased expression or hypermethylation of promoter region. Pathways enriched by differentially regulated genes are noted at the far left, single genes are noted in the circles. These genes are upstream from the pivotal genes in the PI3K-AKT pathway and further regulating single gene expression and other pathways.

Figure 3 Heat map and hierarchical clustering, lung tissue.