Insights on the maturation steps of pulmonary immune responses in neonatal mice

– a transcriptome-based study –

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TABLE OF CONTENT

8. INTRODUCTION

Early life events are critical for educating the immune system and setting an individual on a healthy trajectory. Both innate and adaptive immune responses in the neonate are immature at first, due to the necessity to tolerate maternal antigens during pregnancy to avoid inflammatory responses in the fetus and potential complications like premature birth or abortion[1]. As the neonate grows its immune system has to mature in a series of critical steps that will allow him to fight pathogens, to avoid exacerbated allergic reactions, and to limit the development of chronic inflammatory diseases. Notably, the first maturation steps of the pulmonary immune system are particularly critical in defining one's susceptibility to lung diseases[2].

The development of the immune system starts in the yolk sac at embryonic day 8 (E7) in mice and week 3-4 in humans. Afterwards, it is taken over by the ventral wall of the aorta in the aorta-gonad mesonephros, and starting from week 5, hematopoiesis is initiated in the fetal liver. Extra embryonic hematopoiesis ceases around E12 in mice, week 10-12 in humans. The liver is the main hematopoietic site until birth in mice, and week 20-24 in humans. From the fetal liver, hematopoietic stem cells (HSCs) colonize the fetal thymus and spleen. At this stage, HSCs are expanding, however, they are not generated *de novo*. Just before birth, in mice, and during the second trimester, in humans, a small pool of HSCs colonizes the bone marrow. These bone marrow resident HSCs will be responsible for the maintenance of hematopoiesis throughout life[3,4].

At birth, the airways are exposed for the first time to the external environment and have to adapt quickly. Indeed, *in utero* the exposure to exogenous antigen is very limited. The embryo's cellular immunity is poorly stimulated, and thus suboptimal in neonates, which have to rely mostly on innate responses[5]. In the neonate, both innate and adaptive immune responses are immature: (1) They have less $CD4^+$ and $CD8^+$ T cells, they can build strong Th2 responses but are weak at inducing Th1 responses as the threshold of Th1 stimulation is higher than in adults; (2) there are less dendritic cells, which are less mature and less inclined to promote Th1 responses; (3) regulatory T cells show lung-specific early expansion; (4) antibody production by B cells is impaired[1,6-8]. Overall, the immune system of a healthy neonate has to adapt to the external environment, and both the innate and adaptive responses have to adjust accordingly.

There are still many unanswered questions on how the immune system develops in general, but also specifically in the lungs of neonates. The aim of this project is to discover the characteristics of these immune maturation steps in the lungs early in life, and to determine some of the mechanisms behind this process. More specifically the goals are: (1) to analyze mRNA sequencing data of hematopoietic cells from lungs of naïve mice during development (3, 8, 16 and 45 days of age) in order to identify the most interesting genes and pathways involved in immune maturation, and discover how they differ with age; and (2) to validate and characterize the most intriguing targets experimentally.

METHODS

Mice

BALB/c mice, originally obtained from Charles River Breeding Laboratories (l'Arbrele Cedex, France), were housed under specific pathogen-free conditions and time-mated to obtain offsprings. Time of birth was recorded as day 0 of birth. Animal experiments were performed in accordance with the Cantonal Veterinary Office of Canton de Vaud, Switzerland.

Cell preparation and cell sorting

Splenocytes were obtained by gently disrupting the spleen through a 70 μm cell strainer, followed by extensive washing and red blood cell lysis. Lungs were perfused with phosphate-buffered saline (PBS) and digested in medium with 2 mg/mL Collagenase V (Roche, Rotkreuz, Switzerland) for 45 min and the remaining tissue disrupted through a 70 μm cell strainer, followed by extensive washing and red blood cell lysis. Cells were magnetically sorted either using CD45 MicroBeads (Miltenyi Biotec GmbH, Germany) or an anti-c-kit-biotin antibody (Biolegend, San Diego, CA) with streptavidin MicroBeads (Miltenyi Biotec GmbH). Once labeled with beads following the manufacturers instructions, cells were passed through a LS column (Miltenyi Biotec GmbH) placed on a MACS separator magnet (Miltenyi Biotec GmbH). The flow-through and washes correspond to the negative fraction. The positive population was flushed out mechanically by removing the column from the magnet and firmly applying the plunger supplied with the column.

Gene ontology analysis of lung mRNA

Lung and trachea of a mix of male and female mice were harvested; cells were prepared and sorted using CD45 MicroBeads as described above. The $CD45^+$ and $CD45^-$ population of several mice were pooled at different time points (7 mice at d3 and d8, 5 mice at d16 and 4 mice at d45). The mRNA was sequenced on the miSeq platform (Illumina) of the Genomic Technologies Facility (GTF) of the University of Lausanne. The gene ontology enrichment analysis and visualization tool GOrilla (http://cbl-gorilla.cs.technion.ac.il) was used to define relevant process in the mRNA data set. We entered the list of upregulated, and the one of downregulated genes separately in the gene ontology and visualization tool GOrilla in order to obtain a "process" tree. We then looked closely at all the downstream "process" hits and formulated hypotheses.

Quantification of mRNA levels

50-100mg of lung, spleen and liver tissue, or cells were kept at -20°C in Trizol (Molecular Research Center, Cincinnati, OH). Tissues were homogenized with 5mm stainless-steel beads, in a tissue lyser (TissueLyser, Qiagen). For RNA extraction bromochloropropane (Sigma-aldrich, Buchs, Switzerland) was added on top of the trizol and mixed vigorously. After centrifugation, the acqueous phase was mixed with isopropanol to precipitate the RNA. The pellet was then washed with 70% ethanol. Ethanol was removed, and, once dried, the RNA pellets were solubilized in nuclease-free water. The RNA amounts were measured with a nanodrop (Nanodrop 1000 spectrophotometer, Thermo Scientific). RNA samples (10 μL at 100 ng/μL) were treated with 0.5 U of DNAseI (Invitrogen, Zug, Switzerland) 15 min at room temperature, 1 μL of 25mM ethylenediaminetetraacetic acid (EDTA) solution (Invitrogen) was added to the reaction which was incubated at 65°C for 10 min. Reverse transcription was done using the iScript cDNA kit (Bio-Rad, Hercules, CA) in a cycler at 25°C for 5min, 42°C for 30 min, and finally 5min at 85°C (labcycler, SensQuest). mRNA levels were assessed by quantitative RT-PCR using the following primer sets: MPO-forward: 5'- TGTGGCCAAGGCCTTT CAAT-3', MPO-reverse: 5'- ATAGGCACAGCCACTTGACA-3', EPX-forward: 5'- AAGAGGCGTAA TGGCTTCCT-3', EPX-reverse: 5'- GCCCCACTGCATGAACATAAG-3', Elane-forward: 5'- GAGGC GTGGAGGTCATTTCT-3', Elane-reverse: 5'- GCACTGACCGGAAATTTAGGC-3', MBP-forward: 5'- ACAAAGCTCAGTCAGTTTGCC-3', MBP-reverse: 5'- TGGTTGATTCCCCTGACAGC-3', Cathepsin G-forward: 5'- AGGAGATGAGGCAGGGAAGAT-3', Cathepsin G-reverse: 5'- CTTCTC GCACCAGAAACCCT-3', Beta-actin-forward: 5'-GATCAAGATCATTGCTCCTCCTGA-3', Beta-actin-reverse: 5'- CAGCTCAGTAACAGTCCGCC-3'. 10 μL PCR reactions were set up containing 2 μL of template DNA at a concentration of 50 ng/μL or 100 ng/μL depending on the experiment, 5 μL of SsoAdvanced SYBR Green reaction mix (BioRad), 0.25 μL of each primer at a concentration of 20 μM and 2.5 μL of nuclease-free water. Quantitative PCR was performed on the CFX96 Touch Rea-Time PCR detection System (Bio-Rad) using the following conditions: 1 cycle at 95°C for 2 min, and then 40 cycles at 95°C for 15 s and 60°C for 30 s, followed by a dissociation stage at 65°C for 31 s and cycles of 5 s starting at 65°C, raising 0.5°C per cycle, to obtain melting curves for specificity analysis. After amplification, Cq values were obtained using the CFX ManagerTM software 2.1 (Bio-Rad).

Flow cytometry analysis

Cells were stained for flow cytometry with anti-CD45.2-FITC (BioLegend), anti-Lineage-Pacific Blue (BioLegend), anti-Sca1-PE-Cy7 (BioLegend), anti-ckit-APC (BioLegend). Cells were acquired on LSR II

or Fortessa (BD Biosciences, San Jose, CA). Data was analysed using FlowJo software (Tree Star Inc., Ashland, OR).

Statistical analysis

Ordinary one-way ANOVA with Tukey's multiple comparison test was used to calculate significance levels between experimental groups. Graph generation and statistical analysis was performed using Prism (GraphPad, La Jolla, CA).

RESULTS

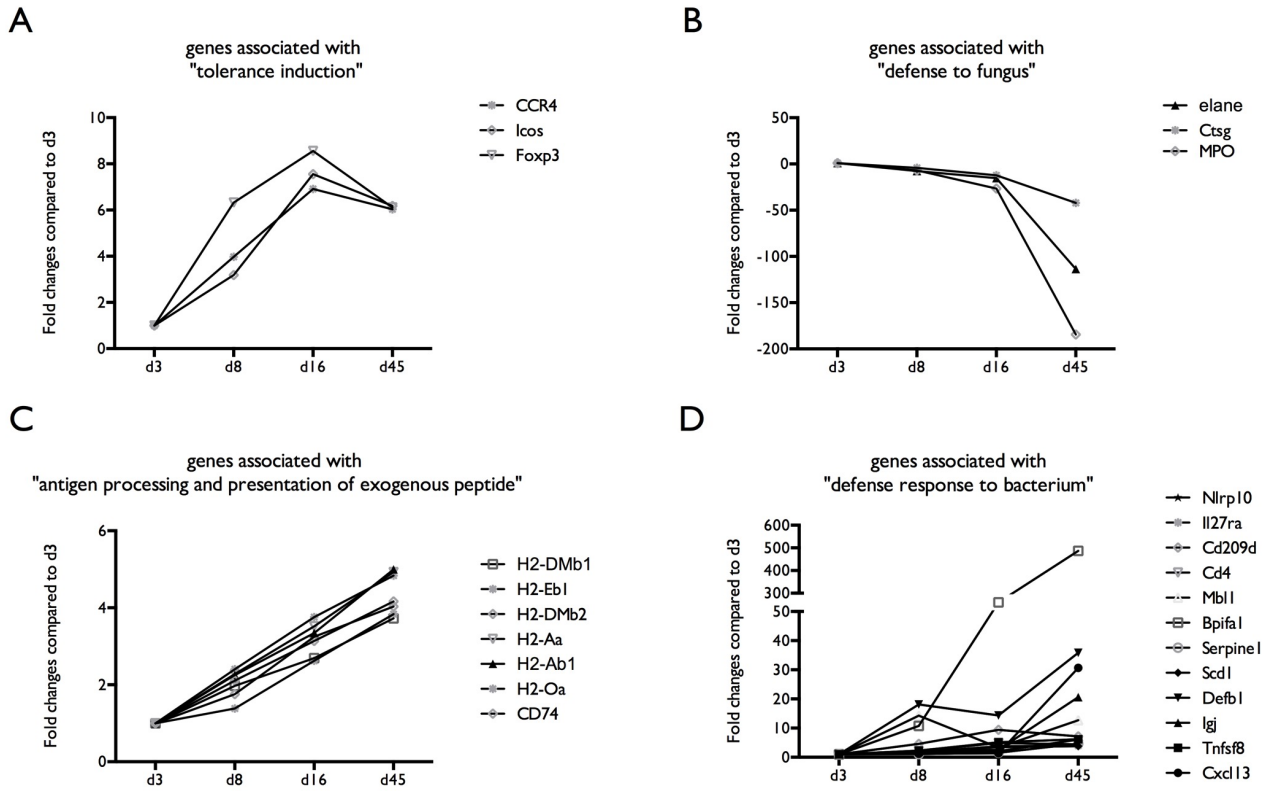
Maturation of the lung immune system is associated with upregulation or downregulation of specific genes, which can be clustered into gene ontology groups. The mRNA sequencing data of $CD45^+$ hematopoietic cells from lungs of mice 3 days, 8 days, 16 days and 45 days old was analyzed using the gene ontology tool GOrilla. We obtained an impressive amount of insights on how the immune system matures in lungs of mice. For more detailed analysis, we decided to select a few examples that seemed particularly relevant to us (**Figure 1**). Genes associated with "tolerance induction", such as the regulatory T cell marker FoxP3[9], are upregulated until d16 and then stabilize by d45 (**Figure1, A**). On the opposite, genes associated with "defense to fungus", including three neutrophil genes[10] (myeloperoxidase (MPO), the elastase Elane, and the protease Cathepsin G (ctsg)), are downregulated as the immune system matures (**Figure 1, B**). For "antigen processing and presentation of exogenous peptide" genes associated with Major Histocompatibility complex - II (MHC-II), there is a linear upregulation as the mice age (**Figure 1, C**). And finally, in the "defense response to bacterium" category, we can see an overall upregulation of the genes with some like bpifa1 coding for a protein which displays antibacterial activity against Gram-negative bacteria[11], being considerably augmented with age, while most others such as the inflammasome associated gene NLRP10[12], have a slower slope of upregulation (**Figure 1, D**). Amongst the highly downregulated genes we also identified two eosinophil-associated genes[13] eosinophil peroxidase (EPX), and the major basic protein (MBP, proteoglycan 2 (prg2)) which were downregulated more than 200 fold between d3 and d45 (293 folds for EPX, and 275 folds for MBP). Together these results indicate that the lung's immune system is undergoing an impressive quantity of maturation steps from birth to adulthood. For the rest of this work, we have decided to focus on granulocytes-associated genes, which are significantly downregulated as the mice age, and are known to be critical players of immunity[1,14].

Neutrophil and eosinophil associated genes are upregulated at birth, and start to be downregulated as the mice age, both locally in the lung and systemically in the spleen and liver. The relative expression of the neutrophil-associated genes: MPO, Elane and ctsg; and of the eosinophil-associated genes: EPX and MBP, were measured in lung, spleen and liver of embryos (E18), neonates (d3), young (d8 and d16), and adult (d45) mice. Both neutrophil (**Figure 2, A-C**) and eosinophil (**Figure 2, D and E**) genes are upregulated at birth and then downregulated as the mice progress to adulthood.

C-kit$^+$ myeloid progenitor cells are present in the lung and spleen of neonatal mice, but not in adults. In adults, myeloid progenitor cells are only present in the bone marrow in adults but we wondered if this was also the case in neonates[4]. To answer this question we stained bone marrow, lung and spleen cells with a panel designed to identify myeloid precursor cells. We first gated on the CD45.2$^+$ lineage$^-$ cells to remove the fully differentiated cells, and then looked at the expression of the hematopoietic stem cell markers Sca-1 and c-kit. Sca-1$^+$c-kit$^+$ are common lymphoid progenitors, whereas Sca-1$^-$c-kit$^+$ cells correspond to the myeloid progenitor population[15]. In the bone marrow of both adults and neonates myeloid progenitor cells were easily identified (data not shown). In lung and spleen of neonates we see a significant increase in the proportion of the Sca-1$^-$c-kit$^+$ myeloid progenitor population (3.7 fold increase in the lung, and 14.4 fold in the spleen) as compared to adult mice, where this population is nearly absent. (**Figure 3**). The same was true for the liver, where neonates have a lot of myeloid progenitor cells, while adults barely have any (data not shown, from Dr. Eva Gollwitzer).

Neutrophil and eosinophil genes are expressed almost exclusively in c-kit$^+$ cells in the lung of neonatal mice, while in the spleen both c-kit$^+$ and c-kit$^-$ cells express them. Neonatal lung and spleen cells were sorted to separate the c-kit$^+$ from the c-kit$^-$ cells to look at the expression of neutrophil and eosinophil genes in the myeloid progenitors (c-kit$^+$) as opposed to fully differentiated cells (c-kit$^-$)[16]. In the neonatal lung all the neutrophil (MPO, Elane, and ctsg) and the eosinophil (EPX and MBP) we looked at were expressed in the c-kit$^+$ fraction of the cells, indicating the presence of a population of granulocyte precursors in the lung (**Figure 4, A**). However, in the spleen neutrophil- and eosinophil-associated genes are expressed in both c-kit$^+$ and c-kit$^-$ populations (**Figure 4,B**).

FIGURES



*<u>Figure 1</u>. **Some genes cluster in gene ontology categories and are either upregulated or downregulated during the maturation of the immune system in the lung, a few examples.** Lungs and trachea of mice were harvested at different time-points (d3, d8, d16 and d45 after birth) and hematopoietic cells ($CD45^+$) were sorted and mRNA was sequenced. Fold changes between the different time-points were analyzed and genes were grouped according to gene ontology clusters. As examples, genes associated with "tolerance induction" **(A)**, "defense to fungus" **(B)**, "antigen processing and presentation of exogenous peptide" **(C)**, and "defense response to bacterium" **(D)** were chosen and presented.*

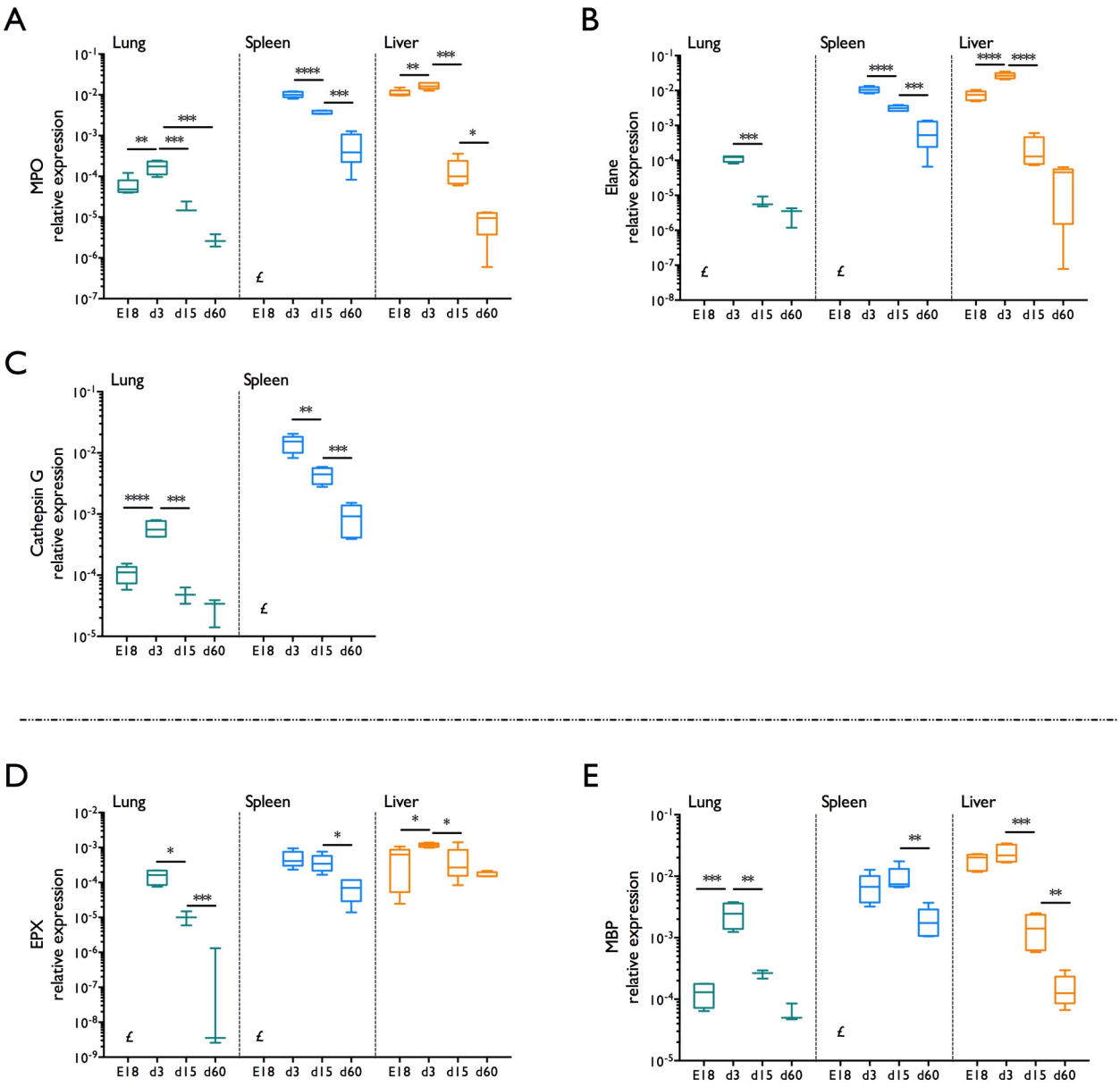


Figure 2. Neutrophil- and eosinophil-associated genes are upregulated at birth, and get downregulated later in life as the immune system matures. *Lung, spleen and liver of mice were harvested at different time-point (embryonic d18 (E18), d3, d15 and d45). mRNA levels in the tissue measured by quantitative RT-PCR. Relative expression of genes as compared to beta-Actin is shown. In the top panel, 3 genes associated with neutrophils: MPO* ***(A)****, Elane* ***(B)****, and Cathepsin G* ***(C)****. In the bottom panel, 2 genes associated with eosinophils: EPX* ***(D)****, and MBP* ***(E)****. (n=3-6 mice per group, "£" means that the data has not been collected for the experimental group, box and whiskers plots are showing the median, the upper and lower quartile as well as the min and max values, ordinary one-way ANOVA with Tukey's multiple comparison test was used for statistical analysis: *p<0.05, **P<0.01, ***P<0.001, ****P<0.0001). Note: Dr. Eva Gollwitzer did the lung quantitative RT-PCR and shared her results.*

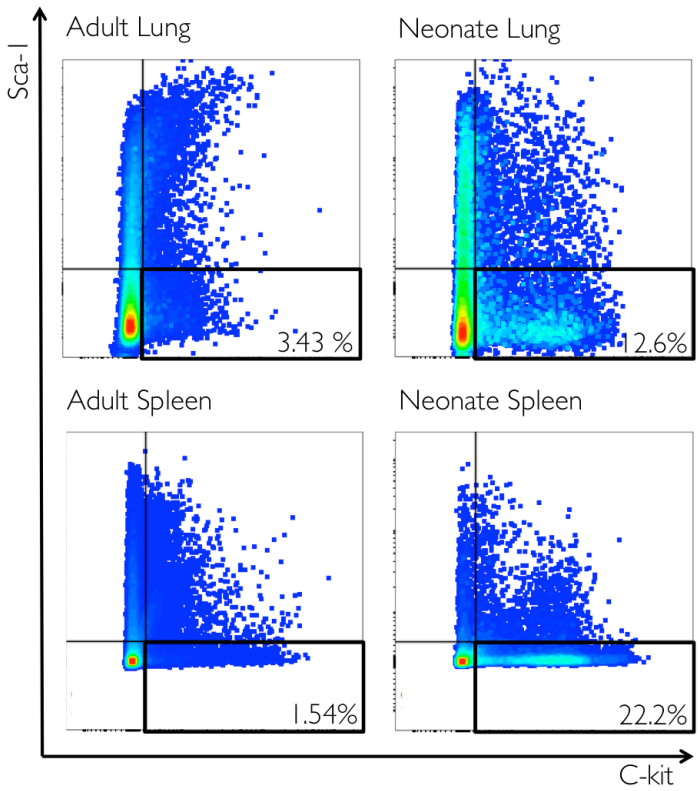


Figure 3. ***Lung and spleen of neonates contain more c-kit$^+$ myeloid progenitor cells than adults'.*** *Lung and spleen of mice were harvested at d3 (neonate) and at d60 (adult). Cells were isolated and stained. Shown is the c-kit versus Sca-1 staining of CD45.2$^+$Lineage$^-$ cells, with the proportion of c-kit$^+$ cells as a percent of the CD45.2$^+$Lineage$^-$ cells.*

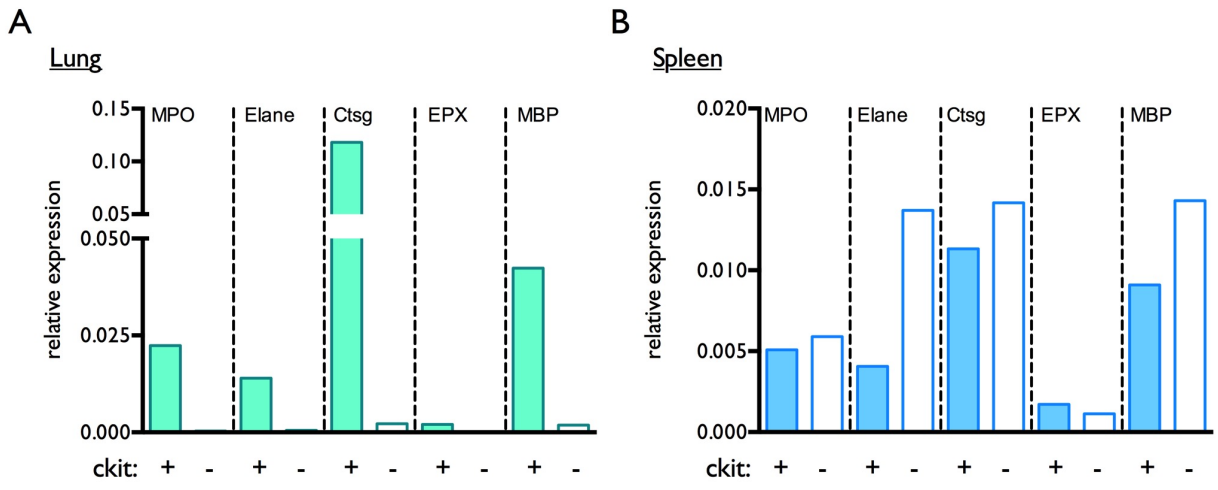


Figure 4. ***In the lung neutrophil- and eosinophil-associated genes are present predominantly in the c-kit$^+$ myeloid progenitor cells of neonates, as opposed to the spleen were these genes are distributed in both c-kit$^+$ and c-kit$^-$ cells.*** *Lung and spleen of neonatal mice (d3, n=4, pooled) were harvested, cells were isolated and sorted to separate the c-kit$^+$ and c-kit$^-$ populations. mRNA levels were measured by quantitative RT-PCR. Relative expression of genes (MPO, Elane, cathepsin G (ctsg), EPX and MBP) as compared to beta-Actin is shown for the lung* ***(A)****, and the spleen* ***(B)****.*

DISCUSSION

This study aimed at determining some of the key steps in the maturation of the immune system in the lung of neonates by analyzing lung mRNA of mice at different ages, and validating a selected immune process experimentally. Our transcriptome analysis of the lungs of mice from birth to adulthood highlighted several interesting immunological phenomena. One of the gene ontology clusters that particularly attracted our attention was a group of neutrophil-associated genes and two eosinophil genes, which were significantly downregulated as mice aged. After validating these hits by quantitative RT-PCR, we found that they were upregulated right after birth to be downregulated again later during development while progressing towards adulthood. The kinetic of granulocyte-associated genes seen in the lung was similar in the spleen and liver, indicating that the effect was systemic rather than local. Characterizing further, we found that c-kit^{+} myeloid progenitor cells were present in the lung, spleen and liver (data not shown, from Dr. Eva Gollwitzer) of neonates, but not in those of adults. Interestingly, the granulocyte genes we looked at were almost exclusively expressed in c-kit^{+} cells in the lung, while in the spleen they were found in both c-kit^{+} and c-kit^{-} cells. Overall these results raise the hypothesis of the presence of myeloid precursor cells in the lung and spleen of neonates that could be able to differentiate into granulocytes directly inside the tissue.

After having analyzed our mRNA sequencing data we decided to focus on neutrophils and eosinophils since their associated mRNA were impressively downregulated (more than 100 folds for MPO, Elane, EPX, and MBP, almost 50 fold for Cathepsin G) in the lungs as mice aged. It has been shown that neutrophils in neonates have both quantitative and qualitative deficiencies, and that their response to infection is impaired[1]. However, the roles of these cell types during the first exposure to a non-sterile environment, and in the establishment of the lung commensal flora is still unknown. Eosinophils intrigue us particularly since previous work in our laboratory had shown that this cell type colonizes neonatal lungs upon house dust mice exposure far more than in adults, correlating with more allergies as well as a higher risk for asthma[17]. The other hits we mentioned were already explored in the literature. It is known that "tolerance induction" is critical to maintain an anti-inflammatory status, and for the induction of tolerance to self-antigens[8]. Genes associated with "antigen processing and presentation of exogenous peptide" are upregulated as antigen-presenting cells become more responsive to their environment with age[7]. The capacity to mount an efficient "defense response to bacteria" is known to increase as the immune system matures[1]. However, it is important to mention that the examples we are showing extracted from our mRNA sequencing bioinformatics analysis only represent a minute quantity of the immune processes involved, and that

many more hypotheses could be generated, which will be explored more thoroughly in future studies[18].

Neutrophil and eosinophil genes are upregulated at birth and downregulated later as the mice age. This kinetic of gene expression is not only seen in the lung, but also in the spleen and the liver. This raises the question of whether this is a systemic effect or if it takes place in the tissue. The next step here will be to use RNA fluorescent *in-situ* hybridization (FISH) microscopy to visualize our genes of interest with RNA probes and see if they are localized in the parenchyma of the tissue, or if they are circulating cells inside blood vessels only passing through different organs. The mechanism involved could potentially be similar to emergency granulopoiesis, which is described as a significant increase in *de novo* production of neutrophils resulting from an enhanced proliferation of myeloid precursor cells in the bone marrow in response to a systemic infection[19]. However, it is also known that neutrophil transmigration to the site of inflammation is impaired in neonates[20], which might imply that some cells, potentially progenitor cells, expressing neutrophil-associated genes reside in the lungs of neonates. On the other hand, neonates seem to have the capacity to produce a large quantity of eosinophils, which have the ability to transmigrate significantly more efficiently than adults'[21]. Even if neutrophils and eosinophils differentiate from the same myeloid progenitor cells, they might not fallow the same maturation steps, tissue distribution, migration properties, and differentiation patterns. Therefore, we will study these two cell types in parallel.

C-kit$^+$ myeloid precursors are present in the lungs and spleen of neonatal mice but not in adults. This is a very interesting finding since it could mean that at birth progenitor cells could be present in the lung ready to differentiate directly inside the tissue as soon as it is exposed to air, and confronted to a non-sterile environment. These cells need to be characterized further; we should define if they are able to differentiate into granulocytes upon proper stimulation. This could be explored e*x vivo* using culture conditions that would favor the differentiation into neutrophils or eosinophils[22,23].

Neutrophil and eosinophil genes are expressed almost exclusively in c-kit$^+$ cells in the lung (but not in the spleen) of neonates, meaning that myeloid progenitors are present and might have the ability to differentiate into granulocytes. A probably comparable phenomenon was described in the context of infected wound healing, where increased survival of neutrophils and c-kit$^+$ progenitor proliferation were shown to correlate with better resolution of the infection[24]. Indeed, having neutrophil progenitor cells inside the tissue allows for an immediate response to efficiently increase the pool of granulocytes directly when and where it is needed. In the context of neonates who have poor

adaptive immunity and rely mostly on innate immunity, this mechanism would be particularly beneficial in case of pathogen exposure. Moreover, it has been shown that neonatal mice are very prone to allergy, in the sense that if they are exposed to house dust mite they have much more eosinophils in their broncho-alveolar lavage fluid than adult mice[17]. This phenomenon could be potentially explained by the presence of eosinophil progenitor cells inside the lungs of neonates, ready to differentiate immediately in the presence of an allergen. Moreover, eosinophils are key players in the defense against parasite infections, and were shown to participate to the clearance of some viruses and bacteria[14]. Overall, the presence of both neutrophil and eosinophil progenitor cells in the lung of neonatal mice would allow for a very efficient and direct induction of immune reactions when suddenly confronted to allergens and pathogens from the external environment.

In conclusion, our data suggests that neutrophils and eosinophils are upregulated very early in life and that they might originate from c-kit^{+} myeloid progenitor cells that could differentiate directly in the lung tissue of neonates. At this stage, our findings are just enough to formulate a hypothesis, but we believe it should be pursued further. Such studies, based on experimentally validated candidates pre-screened with transcriptome analysis can play a key role in our understanding of how the immune system matures, and we think that many more exciting hypotheses can be teased out of our lung RNA sequencing data set.

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