Characterization of two promising experimental tools to further improve the functional analysis of neutrophils

Ph.D. Thesis

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Budapest

2021

Introduction

Neutrophilic granulocytes (neutrophils) are the most abundant circulating leukocytes in humans and a prominent leukocyte population in mice. Neutrophils are essential players in innate defence mechanisms against invading pathogens, but they have also long been known to play an important role in the formation of acute inflammatory responses. Since their inappropriate activation often leads to various diseases like autoimmune and inflammatory diseases or even cancer development it is essential to gather as much information on their biology as possible.

Effector mechanisms of neutrophil responses to pathogen burden have been targets of pharmacological manipulation in the recent years with promising results. However great scientific progress in the field is very much limited by the fact that neutrophils are short-lived, terminally differentiated cells. These characteristics of the neutrophils make their long-term maintenance, culture, expansion, and genetic manipulation in vitro nearly impossible. Although there are several myeloid leukemia cell lines with a potential to differentiate into neutrophil-like cells, most of these lack very important features of neutrophils (e.g.: they cannot carry out phagocytosis or they do not release granule proteins, etc.) so they cannot be used in extensive functional studies. Instead of neutrophil-like cell lines researchers can use primary neutrophils freshly isolated from blood or bone marrow, but these neutrophils are only viable for a couple of hours and can be used only in short-term experiments. The most widely used tool to test the effect of genetic manipulations on neutrophil function is to use transgenic mice carrying germline mutations. However, generation and maintenance of these mouse strains is very difficult and highly expensive, not to mention that studies on these mouse models in vitro are also

limited by the small number of neutrophils that can be obtained from these animals.

We were hoping to overcome the above-mentioned obstacles of neutrophil research, therefore I was focusing on characterising two different approaches and tools that could be useful to study neutrophil cell functions.

One of the tools is the so called HoxB8 myeloid progenitor cell line. HoxB8 cells are murine progenitor cells immortalised via retroviral transduction, such that they retain their differentiation potential to develop into various myeloid cell types. In my thesis, I am going to describe the HoxB8 cell line focusing on how the so-called HoxB8 neutrophils can differentiate from the immortalised HoxB8 progenitors. Then I am going to characterise the effector functions of HoxB8 neutrophils in various in vitro experimental conditions. After that I am going to explain the possibility of using the HoxB8 cells in vivo by producing so-called HoxB8 chimeras. Utilizing these chimeric mice in various acute and chronic inflammatory mouse models I would like to demonstrate that HoxB8 neutrophils can play a crucial role in disease development just like normal neutrophils do.

Apart from the characterisation of the HoxB8 system, I am also going to describe some aspects of the $Mcl1^{\Delta Myelo}$ transgenic mouse model, which is a very promising neutropenia mouse model that can also be a promising tool in neutrophil-related in vivo studies.

Objectives

Our objectives were to answer the following questions:

1. Are we able to generate immortalized HoxB8 progenitor cells?

2. Can we differentiate HoxB8 neutrophils from the HoxB8 progenitor cultures and characterize the effector functions of these cells in vitro.

3. Are we able to find a suitable approach to test the effector functions of HoxB8 neutrophils in vivo, both on a single-cell and whole neutrophil population-based level?

4. Characterizing the effects of *Mcl1* deletion on the leukocyte populations in various tissues of $Mcl1^{\Delta Myelo}$ mice and reassuring the neutrophil-specificity of the mouse model.

Methods

Animals

Wild-type C57BL/6 mice carrying the CD45.2 allele and congenic (B6.SJL-*Ptprc*^{a}) strain (referred to as BoyJ mice) carrying the CD45.1 allele on the C57BL/6 genetic background were purchased from The Jackson Laboratory. Mice carrying the *Mcl1*$^{\mathrm{tm1Ywh}}$ (*Mcl1*$^{\mathrm{flox}}$) floxed allele of the Mcl-1–encoding gene were crossed to mice carrying the *Lyz2*$^{\mathrm{tm1(cre)Ifo}}$ (*Lyz2*$^{\mathrm{Cre}}$) knock-in strain expressing the Cre recombinase in the myeloid compartment to generate *Lyz2*$^{\mathrm{Cre/Cre}}$*Mcl1*$^{\mathrm{flox/flox}}$ mutants (referred to as *Mcl1*$^{\Delta\mathrm{Myelo}}$ mice). Other mouse strains that we used for the experiments: *Syk*^{tm1Tyb} (referred to as Syk$^{+/-}$), Tg(TcraR28,TcrbR28)KRNDim (KRN), NOD (Non-obese diabetic). All mice were on the C57BL/6 genetic background. Our experiments were approved by the Animal Experimentation Review Board of the Semmelweis University.

Generation of HoxB8 chimeras

To generate HoxB8 chimeras, recipients carrying the CD45.1 allele on the C57BL/6 genetic background, also known as BoyJ mice, were lethally irradiated followed by single or multiple intravenous injections of in vitro cultured HoxB8 progenitors.

Cell cultures and flow cytometry

Generation of immortalized HoxB8 progenitor cells. HEK-293T cells were transfected with pMSCVneo-ER-Hoxb8 retroviral and pCL-Eco packaging vectors. Virus-containing supernatants were harvested 48 h after transfection. Bone marrow cells of 8-10-week-old mice or fetal liver cells of embryos (embryonic day 15-16) were harvested, and progenitor cells were enriched by a Histopaque-1083 gradient. Progenitor cells were collected and were further

cultured in SCF (Stem Cell Factor)- containing cell supernatant, recombinant murine IL-3 and murine IL-6 containing media for 72 hours. Progenitor cells were transduced with the retrovirus particles and grown in SCF cell supernatant and β-estradiol containing cell culture media. The first immortalized cells emerged in 10-14 days after retroviral transduction.

In vitro culturing and differentiation of HoxB8 neutrophils. To elicit neutrophilic differentiation of HoxB8 progenitors, β-estradiol was removed from the HoxB8 progenitor culture. HoxB8 progenitor cells were counted, and the cells were further cultured in HoxB8 neutrophil-growth media which contained mouse G-CSF (Granulocyte Colony Stimulating Factor) at appropriate concentration, typically for 5 days.

Flow cytometry. The different cell populations in vitro and in vivo were identified within their typical forward and side scatter gates combined with staining for their specific cell surface markers. Dead cells were identified using the LIVE/DEAD Fixable Far Red dead cell staining kit. Peripheral blood samples were obtained from tail vein incision. Bone marrow and spleen samples for flow cytometric analysis were obtained by flushing the bone marrow or crushing the spleen of the mice followed by red blood cell lysis.

In vitro functional assays

Adherent respiratory burst. Adherent respiratory burst was measured by a cytochrome c reduction test using PMA (Phorbol 12-myristate 13-acetate) or immobilized immune complex stimulation.

Cell migration. For in vitro migration Transwell-system was used, and migration was measured towards chemoattractant such as fMLP (N-Formylmethionyl-leucyl-phenylalanine) or MIP-2 (Macrophage Inflammatory

Protein 2). Migrated neutrophil counts were determined by an acid phosphatase assay.

Phagocytosis assay. For the analysis of in vitro phagocytosis, HoxB8 cells were incubated with non-opsonized or mouse serum-opsonized GFP-expressing USA300 *Staphylococcus aureus* or Alexa Fluor 488-labelled *Candida albicans* (SC5314). Using a flow cytometer cells in the accurate call gate for HoxB8 progenitors and HoxB8 neutrophils were investigated for green fluorescence signal.

In vivo functional assays

Thioglycollate-induced peritonitis. Peritonitis was induced by intraperitoneal injection of thioglycollate or PBS as control. After peritoneal lavage cell were analyzed in flow cytometer.

In vivo phagocytosis. Mice were injected intraperitoneally with thioglycollate. Then heat-killed and AF488-stained *C. albicans* particles were inject into the peritoneum after control or Latrunculin A treatment. After peritoneal lavage cell were analyzed for green fluorescens in flow cytometer combined with Trypan Blue quenching.

Reverse passive Arthus reaction. Ears of the mice were treated separately with anti-ovalbumin whole serum or control serum. Then mice were injected intravenously with ovalbumin followed by the intravenous injection of Evans blue to visualize increased infiltration and edema formation. Ear thickness, cellular infiltration, cytokine and chemokine production in the ears were measured with ELISA method.

K/B×N serum transfer arthritis. The autoantibody-mediated arthritis was induced by intraperitoneal injection of K/B×N (arthritic) or B×N (control)

serum, followed by daily scoring of clinical signs of arthritis and measurement of ankle thickness.

Presentation of data and statistical analysis

Experiments were performed three or more times with two or more mice/genotype/treatment. Statistical analyses were carried out by the Statistica software (StatSoft) using one-way ANOVA for evaluation of superoxide production and two-way (factorial) ANOVA for all other experiments. In case of kinetic assays, area under the curve (AUC) was used for statistical analysis. p values below 0.05 were considered statistically significant.

Results

Characterization of neutrophil-like cells differentiated from HoxB8 progenitors

In vitro differentiation of HoxB8 progenitors towards neutrophil-like cells. We have generated conditionally HoxB8-transduced progenitors by retroviral transduction of wild-type mouse bone marrow cells with an ER-HoxB8 fusion protein. To induce neutrophilic differentiation of HoxB8 progenitors, the cells were cultured in the presence of SCF-containing supernatant and G-CSF without addition of β-estradiol. Cells cultured under such conditions for 5 days (referred to as HoxB8 neutrophils) showed a morphology similar to that of mouse neutrophils with condensed and ring-shaped nuclei and increased expression of neutrophil specific markers such as Ly6G and certain cell surface receptors involved in the functional activation of neutrophils.

In vitro functions of HoxB8 neutrophils. HoxB8 neutrophils mounted a robust respiratory burst upon stimulation with PMA, a nonphysiological stimulator of neutrophils, and also when plated on immobilized IgG immune complexes, a model of autoantibody-induced, Fc-receptor-dependent neutrophil activation. HoxB8 neutrophils were able to migrate towards both fMLP and MIP-2. HoxB8 neutrophils mounted a robust phagocytosis response both of serum-opsonized bacteria and fungi, whereas none of the above-mentioned functional responses were detectable using HoxB8 progenitor cells.

In vivo transplantation of HoxB8 progenitors. Since HoxB8 neutrophils seemed to mount various effector functions in vitro, we hoped to

test their capacities in vivo as well. Therefore, we decided to transplant HoxB8 progenitors into lethally irradiated recipient mice, generating the so-called HoxB8 chimeras. Flow cytometric profile of such chimeras showed that 5 days after irradiation their circulating neutrophils (Ly6G-positive cells) consisted practically exclusively of donor-derived HoxB8 cells. Furthermore, the neutrophil compartment of bone marrow and spleen in these chimeras were also completely of donor origin. Due to a single transplantation of HoxB8 progenitors, this HoxB8 neutrophil population was detectable in the circulation of the chimeras for approximately 3 days. However, if multiple repeated transplantations of HoxB8 progenitors were executed, the HoxB8 neutrophil population could be maintained in the circulation of the HoxB8 chimeras for a prolonged time period.

Phenotypic and functional characterization of in vivo differentiated HoxB8 neutrophils. In vivo generated HoxB8 neutrophils have mostly normal size (FSc) and granularity (SSc) compared with neutrophils of intact mice. Neutrophils from HoxB8 chimeras and intact mice showed a similar peak Ly6G and other neutrophil-specific cell surface marker level, suggesting nearly normal in vivo differentiation of HoxB8 progenitors towards neutrophils in vivo.

Single-cell-based effector functions of HoxB8 neutrophils in vivo. Thioglycollate triggered a robust infiltration of neutrophils into the inflamed peritoneum of HoxB8 chimeras. This infiltrating neutrophil population consisted practically entirely of donor-derived HoxB8 cells in line with the practically complete absence of recipient-derived circulating neutrophils in these chimeras. Once sterile peritonitis was triggered and HoxB8 chimeras were

injected with *C. albicans*, we could detect that a substantial percentage of HoxB8 neutrophils phagocytosed the heat-killed C. albicans cells. This response was practically completely blocked by latrunculin A, confirming the role of the cytoskeletal machinery in the phagocytic response.

Whole neutrophil population-based responses of in vivo differentiated HoxB8 neutrophils.

Reverse passive Arthus reaction: Edema formation indicated by blue staining of the ear (tissue accumulation of Evans Blue) was triggered by anti-Ova but not control treatment in HoxB8 chimeras, similar to intact animals. A substantial neutrophil infiltration was observed in the anti-Ova-treated ears of HoxB8 chimeras but not in the contralateral control ears or in the ears of non-transplanted control animals. Similarly, anti-Ova treatment triggered the accumulation of the pro-inflammatory cytokine IL-1β and the CXC chemokine MIP-2 in HoxB8-chimeras but not in irradiated non-transplanted mice.

Autoantibody-induced arthritis in HoxB8 chimeras: Arthritogenic but not control serum injection triggered robust arthritis in intact C57BL/6 mice. In contrast, no signs of arthritis could be observed in irradiated non-transplanted mice. Importantly, however, arthritis development could also be observed in HoxB8 chimeras, which was similar to, or in the case of ankle thickening, even slightly more pronounced than, that in intact mice.

Validation of the HoxB8 system using Syk$^{-/-}$ HoxB8 chimeras. Since we had very promising outcomes from the previously mentioned experiments, we wanted to further verify the accuracy of the HoxB8 system. Therefore, we used a *Syk* knock out HoxB8 progenitor cell line yielding *Syk*$^{-/-}$ HoxB8 neutrophils, which according to our previously reported observations, were

expected to show deficiency in many neutrophil-related effector functions. We found that even though *Syk*$^{-/-}$ HoxB8 neutrophils differentiated in vitro expressed the typical neutrophil-specific cell surface markers, but these cells were unable to produce reactive oxygen species upon stimulated with PMA. We observed practically the same potential of WT and *Syk*$^{-/-}$ HoxB8 progenitors to differentiate into HoxB8 neutrophils in vivo as well. However, *Syk*$^{-/-}$ HoxB8 chimeras were completely protected from autoantibody-induced serum transfer arthritis upon arthritic serum treatment, further confirming that HoxB8 neutrophils differentiated from the immortalized HoxB8 progenitors are just as functionally active as any bone marrow-derived neutrophils in the circulation.

Characterization of *Mcl1*$^{\Delta Myelo}$ mice

Leukocyte populations in the bone marrow of Mcl1ΔMyelo mice. The number of Ly6G^{high} neutrophil population in the bone marrow was strongly reduced in the *Mcl1*$^{\Delta Myelo}$ animals. This phenomenon was further confirmed by cytospin preparation and Giemsa-May-Grünwald dye staining of bone marrow cells. We could see a strong reduction in the number of cells with neutrophil-like donut-shaped nuclear morphology. However, the immature neutrophil populations with Ly6G$^{med/dim}$ expression were not reduced, suggesting that the *Mcl1*$^{\Delta Myelo}$ mutation does not eradicate the myeloid progenitors all together, but leaves behind an early neutrophil-lineage-like compartment. Further analysis of bone marrow did not reveal any difference in monocyte, macrophage, dendritic cell and T cell counts between wild type and *Mcl1*$^{\Delta Myelo}$ mice either. However, there was a clear reduction in bone marrow B cell counts likely due to a disturbed bone marrow B cell niche.

Leukocyte populations in the spleen of Mcl1$^{\Delta Myelo}$ mice. Splenic neutrophil numbers were strongly reduced in *Mcl1*$^{\Delta Myelo}$ animals. However, the number of splenic T or B cells, macrophages or dendritic cells was not reduced. In fact, we found an increased number of splenic macrophages in *Mcl1*$^{\Delta Myelo}$ animals, and we believe that the increased macrophage number is related to splenomegaly of the mice, reflecting the fact that macrophages represent one of the predominant cell types in this organ.

Analysis of the differentiation of macrophages from Mcl1$^{\Delta Myelo}$ mice in vitro. Since we found that *Mcl1* deletion caused increased macrophage numbers in the spleen of the animals, we have also tested the in vitro differentiation of macrophages from *Mcl1*$^{\Delta Myelo}$ bone marrow cells to test if the gene mutation had any side effects on the macrophage biology apart from the arrest in neutrophil differentiation. We did not observe any difference between the number of bone marrow-derived macrophages generated from wild type or *Mcl1*$^{\Delta Myelo}$ bone marrow in vitro. On the other hand, PCR analysis of genomic DNA confirmed deletion of the *Mcl1*^{flox} allele in bone marrow-derived macrophage cultures while only a slight deletion (likely due to the presence of tissue macrophages or osteoclasts) was seen in tail biopsy samples. These results indicated that the normal number and differentiation capacity of macrophages were retained even though Mcl-1 was effectively deleted from these cells as well.

Conclusions

Based on the results of our studies, I summarize my conclusions in the following points:

1. We were able to generate HoxB8 progenitors of various genotypes both from progenitor cells derived of the bone marrow or fetal liver of mice. We could not detect any phenotypical differences between HoxB8 progenitors of different origin. HoxB8 progenitor cells could be kept in culturing media supplemented with SCF and estrogen, for long periods of time. During culturing, HoxB8 progenitors were able to divide and proliferate in a rapid manner, without significant apoptosis along the way.
2. With the right culturing protocol (estrogen withdrawal and G-CSF addition to the culture), HoxB8 progenitors differentiated into HoxB8 neutrophils in my hands. We could generate basically unlimited numbers of HoxB8 neutrophils in vitro. HoxB8 neutrophils differentiated in vitro resembled neutrophils originating from WT mice both phenotypically and functionally.
3. We were able to generate HoxB8 chimeras for the in vivo characterization of the HoxB8 cells. HoxB8 progenitors differentiated into mature neutrophil-like cells (the HoxB8 neutrophils) with a strong, convincing neutrophilic nature. The number of HoxB8 neutrophils could be kept steady for up to 10 days with repeated transplantation of HoxB8 progenitors into lethally irradiated recipients.
4. HoxB8 neutrophils can develop all tested functional properties of circulating neutrophils both at a single-cell and whole neutrophil population-based level. Their functional activity is comparable to

neutrophils of intact animals, and they even contribute to the development of complex inflammatory conditions, as well.

5. The deletion of *Mcl1* does not affect the cell number of the majority of the leukocyte populations in the bone marrow and the spleen of $Mcl1^{\Delta Myelo}$ mice, except for a drastic reduction in neutrophil counts.

Publications

PUBLICATIONS RELEVANT TO THE DISSERTATION:

I. **Orosz A,** Walzog B, Mócsai A. (2021) In Vivo Functions of Mouse Neutrophils Derived from HoxB8-Transduced Conditionally Immortalized Myeloid Progenitors. *J Immunol*, 206 (2): 432-445.

I.F.: 4,886

II. Csepregi JZ*, **Orosz A***, Zajta E, Kása O, Németh T, Simon E, Fodor S, Csonka K, Barátki BL, Kövesdi D, He YW, Gácser A, Mócsai A. (2018) Myeloid-specific deletion of Mcl-1 yields severely neutropenic mice that survive and breed in homozygous form. *J Immunol*, 201: 3793-3803. (**shared first authors*)

I.F.: 4,718

OTHER PUBLICATIONS:

I. Ayoglu B., Szarka E, Huber K, **Orosz A,** Babos F, Magyar A, Hudecz F, Rojkovich B, Gáti T, Nagy Gy, Schwenk J, Sármay G, Prech J, Nilsson P, Papp K (2014) Bead arrays for antibody and complement profiling reveal joint contribution of antibody isotypes to C3 deposition. *PLos ONE,*9(5): e96403

I.F.: 2,776