Characterization and application of the neutropenic *Mcl-1*$^{\Delta Myelo}$ mouse strain in inflammatory disease models

Ph.D. Theses

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2020

Introduction

Genetically manipulated mice lacking a certain hematopoetic lineage have strongly contributed to the understanding of immune and inflammatory processes in health and disease.

Neutrophils are critically involved in the innate immune response during the host protection against pathogens, but they also contribute to tissue damage upon inappropriate activation of the cells. The neutrophils can recognize pathogens and the inflammatory environment, and after leaving the circulation, they migrate to the site of inflammation and eliminate the microorganisms.

The lack of appropriate neutrophil depletion antibodies, drugs or neutropenic mouse strains makes it difficult to investigate the role of neutrophils. Several mouse strains with reduced neutrophil counts have been described previously, but all of them have some substantial limitations: poor specificity, partial neutrophil deficiency, the mutant mice suffer from general health problems, or the limited survival of the affected animals.

Taken in consideration that there is no suitable neutrophil-deficient mouse model for the *in vivo* analysis of neutrophil function in normal and pathological conditions, I wanted to provide a solution to this problem in my Ph.D. work.

The survival of neutrophils is regulated by several pro- and antiapoptotic proteins. The Mcl-1 antiapoptotic protein is essential for the

neutrophil survival but does not play a key role in monocyte/macrophage survival. Our initial hypothesis was that deletion of the Mcl-1 protein from myeloid cells results in a neutropenic mouse strain. In the first part of my Ph.D. work, I characterized this mouse strain and applied in a known neutrophil-dependent autoantibody-induced arthritis model. In the second part of my work, using the mouse strain mentioned above, I investigated the role of neutrophils in the phases of contact hypersensitivity model.

Objectives

Our objectives were to answer the following questions:

1. Within the detailed characterization of the *Mcl1*$^{\Delta Myelo}$ neutropenic mouse model, we were interested in how myeloid-specific deletion of Mcl-1 affects:

- the number of neutrophils under normal and sterile inflammatory conditions?
- the number of other leukocyte populations in the peripheral blood?
- survival, appearance, and breeding of the mice?
- protection of the mice against autoantibody-induced arthritis?
- the susceptibility of the animals to bacterial and fungal infections?

2. How does neutrophil-specific deletion of Mcl-1 affect:

- the number of neutrophil granulocytes and other leukocyte populations in the peripheral blood?
- survival and breeding of the mice?

3. What changes characterize the peripheral blood leukocyte populations and the autoantibody-induced arthritis in G-CSF receptor-deficient mice?
4. Which phase of the contact hypersensitivity disease model requires the neutrophils? Does the *Lyz2*$^{Cre/Cre}$ mutation affect the development of the inflammatory response?

Methods

Animals

The genetically modified mouse strains.

Mice carrying the *Mcl1*^{tm1Ywh} (*Mcl1*^{flox}) floxed allele of the Mcl-1–encoding gene were crossed to mice carrying the *Lyz2*$^{tm1(cre)Ifo}$ (*Lyz2*^{Cre}) knock-in strain expressing the Cre recombinase in the myeloid compartment to generate *Lyz2*$^{Cre/Cre}$*Mcl1*$^{flox/flox}$ mutants (referred to as *Mcl1*$^{\Delta Myelo}$ mice). Other mouse strains that we used for the experiments: *MRP8*-Cre*Mcl1*$^{flox/flox}$ (*Mcl1*$^{\Delta PMN}$), Csf3r^{tm1Link} (*Csf3r*$^{-/-}$), Tg(TcraR28,TcrbR28)KRNDim (KRN). All mice were on the C57BL/6 genetic background. Our experiments were approved by the Animal Experimentation Review Board of the Semmelweis University or the University of Szeged.

Preparation of the cells and flow cytometry analysis

Peripheral blood. We used peripheral blood samples taken from the tail vein with heparinized pipette tips, to analyze the leukocyte populations. After washing and centrifugation, the cells were stained with the fluorochrome-conjugated antibodies.

Flow cytometry. The different leukocyte populations were identified within their typical forward and side scatter gates as follows: neutrophils as CD11b^{+}Ly6G^{+}Siglec-F^{-}, monocytes as CD11b^{+}Ly6G^{-}Siglec-F^{-}, eosinophils as Ly6G^{-}Siglec-F^{+}, T-cells (CD3^{+}), B-cells (B220^{+}).

Survival, fertility, and weight measurement

An online database was used for the analysis of the survival, fertility, and breeding behavior of our mice. Body weight of male and female mice was measured once weekly from the age of 2 week.

K/B×N serum transfer arthritis

The autoantibody-mediated arthritis was induced by intraperitoneal injection of 300 µl K/B×N (arthritic) or B×N (control) serum, followed by daily scoring of clinical signs of arthritis and measurement of ankle thickness for 2 weeks.

Contact hypersensitivity model (CHS)

CHS model. 2,4,6-trinitrochlorobenzene (TNCB) dissolved in acetone was used as contact allergen to induce CHS. For sensitization, the mice were treated with 3% TNCB or acetone as control. Five days after sensitization, the initial ear thickness of the mice was measured, then the mice were challenged by application of 1% TNCB on both ears. 24 hours later the ear thickness was measured. The increase of the ear thickness provided information on the extent of the inflammatory response.

Passive CHS model. For the passive (adoptive transfer) CHS model, the donor mice were sensitized with 3% TNCB. Five days after sensitization, the mice were euthanized, the lymph nodes were collected, and a single-cell suspension was prepared. The lymph node cells were transferred by intravenous injection to the naïve recipient mice. Directly after the injection the initial ear thickness was measure and the animals were challenged with 1% TNCB on both ears. After 24 hours, the ear thickness increase was measured.

In vivo infection models

The bacterial strain *Staphylococcus aureus* and the fungal strain *Candida albicans* were used to perform the infection experiments. The survival of the animals and the colonization of the pathogens were monitored in both models. Bacterial and fungal burdens were determined in blood, peritoneal and homogenised organ samples by a conventional CFU (colony forming unit) counting method 12 h post infection.

Presentation of data and statistical analysis

Experiments were performed three or more times with two or more mice/genotype/treatment. We used StatSoft Statistica software for statistical analysis. The analysis of the experimental data was performed by Student's *t* test, two-way factorial ANOVA, or Mann-Whitney U test. Survival studies were analyzed by the Kaplan-Meier method and log-rank statistics. A p value $< 0,05$ was considered statistically significant.

Results

Myeloid-specific deletion of Mcl-1 leads to severe neutropenia. While in the peripheral blood of wild type (WT) animals we could detect a distinct neutrophil population, these cells were almost completely missing from the *Mcl1*$^{\Delta Myelo}$ mice (98,1% reduction). We also tested whether the Mcl1$^{\Delta Myelo}$ mutation had any effect on other leukocyte populations, and we have found that in the *Mcl1*$^{\Delta Myelo}$ mice there was no difference in the circulating monocyte, eosinophil and B-cell counts, while the number of T-cells showed a slight increase compared to WT animals. Upon thioglycollate induced peritonitis a robust neutrophil infiltration could be detected in the WT animals, while in the *Mcl1*$^{\Delta Myelo}$ mice we could not detect any reasonable neutrophil accumulation in the peritoneum. This indicates that the severe neutrophil deficiency of the Mcl1$^{\Delta Myelo}$ animals is still detectable upon inflammatory conditions as well.

Survival and breeding of Mcl1$^{\Delta Myelo}$ mice. To our surprise the survival of the *Mcl1*$^{\Delta Myelo}$ mice in SPF conditions was only slightly, however significantly lower than of their WT counterparts. Despite the lower overall productivity when *Mcl1*$^{\Delta Myelo}$ females were used, breeding of these animals in homozygous form still yielded a comparable number of offspring to the WT animals. Overall, the moderate reduction in the breeding capacity of the *Mcl1*$^{\Delta Myelo}$ mice was not substantially more

severe than what is usually observed during breeding of other genetically manipulated mice.

Mcl1$^{\Delta Myelo}$ mice are protected in autoantibody-induced arthritis. We tested the functional relevance of *Mcl1*$^{\Delta Myelo}$ mice as a neutropenia mice model in an autoantibody-induced, neutrophil-dependent *in vivo* inflammation model – the K/B×N serum transfer arthritis. Arthritic serum injection into WT animals resulted in the development of severe arthritis, while the *Mcl1*$^{\Delta Myelo}$ mice seemed to be completely protected in the same circumstance.

Mcl1$^{\Delta Myelo}$ mice are highly susceptible to bacterial and fungal infections. We exposed the *Mcl1*$^{\Delta Myelo}$ mice to systemic *Staphylococcus aureus* or *Candida albicans* infections, respectively. While WT animals were able to successfully fight the intraperitoneal injection of *S. aureus*, more than 80% of *Mcl1*$^{\Delta Myelo}$ mice succumbed to the same infectious burden and died within 2 days after injection. Intravenous injection of *C. albicans* caused the death of 27% of the WT animals, while the same infection killed 95% of the *Mcl1*$^{\Delta Myelo}$ mice very rapidly. Altogether, *Mcl1*$^{\Delta Myelo}$ mice are highly susceptible to infectious challenges likely because of the malfunctioning neutrophil mediated elimination of the pathogens.

Neutrophil-specific deletion of Mcl-1

To test the effect of *Mcl-1* deletion in a more neutrophil-specific manner, we used the MRP8-Cre*Mcl1*$^{flox/flox}$ (*Mcl1*$^{\Delta PMN}$) mice, where *Mcl-1* expression is deficient in the neutrophil compartment. In these mice

Ly6G-positive cells were almost completely absent, leaving the mice 99,1% neutropenic. The number of other leukocytes (i.e.: monocytes, eosinophils, B- and T cells) were not affected in the *Mcl1*$^{\Delta PMN}$ mice. Analysis of the survival of *Mcl1*$^{\Delta PMN}$ mice revealed a quite high death amongst these animals with only 30% survival at 12 months of age. *Mcl1*$^{\Delta PMN}$ mice were smaller in size, and showed poor overall phenotype compared to their WT littermates. Upon homozygous mating *Mcl1*$^{\Delta PMN}$ mice also showed a very poor breeding productivity: only 14% of the breeding pairs had offspring in total.

Neutropenia in G-CSF receptor-deficient mice

Using flow cytometry, we found a partial neutropenia in *Csf3r*$^{-/-}$ mice. The Ly6G positive neutrophil population was detectable in the peripheral blood of these mice, but the overall neutrophil counts were moderately, though significantly lower than in the WT animals. The *Csf3r*$^{-/-}$ mutation did not affect the size of other circulating leukocyte populations. Testing the effect of the autoantibody-mediated K/B×N serum-transfer arthritis on these animals revealed that *Csf3r*$^{-/-}$ mice are highly protected against inflammatory arthritis. Altogether this implied that even a moderate neutropenia is sufficient to prohibit the development of this inflammatory disease.

Contact hypersensitivity in neutropenic mouse models

According to our previous observations, contact hypersensitivity (CHS), which is the *in vivo* model for human allergic contact dermatitis, was highly deficient in *Mcl1*$^{\Delta Myelo}$ mice. We proved that this deficiency is not

related to the deletion of the Lysozyme M coding *Lyz2* gene, but rather the lack of neutrophil granulocytes itself.

Roles of neutrophil granulocytes in the sensitization phase of CHS. Lymph node cells from mice sensitized with TNCB were able to transfer sensitization to naïve WT recipients. Meanwhile if the sensitization was done in neutropenic *Mcl1*$^{\Delta Myelo}$ mice the recipients did not show the inflammatory response, indicating that neutrophils play important role in the sensitization phase of contact hypersensitivity.

Roles of neutrophil granulocytes in the elicitation phase of CHS. Lymph node cells from WT donors sensitized with TNCB were able to transfer sensitization to naïve WT recipients. However, if the recipients were of the *Mcl1*$^{\Delta Myelo}$ genotype we could not detect the inflammatory CHS response, suggesting that neutrophils play important role in the elicitation phase of contact hypersensitivity.

Conclusion

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

1. The myeloid-specific deletion (*Mcl1*$^{\Delta Myelo}$ mutation) of Mcl-1 anti-apoptotic protein greatly decreased the overall number of neutrophil granulocytes. Meanwhile the size of other leukocyte populations was not affected in the Mcl1$^{\Delta Myelo}$ mice. Despite of their severe neutropenia, *Mcl1*$^{\Delta Myelo}$ mice can survive for prolonged periods and are able to breed in homozygous form. *Mcl1*$^{\Delta Myelo}$ mice seemed to be completely protected in neutrophil-mediated K/B×N serum-transfer arthritis. These mice showed high susceptibility upon bacterial (*S. aureus*) and fungal (*C. albicans*) infections. Altogether, the *Mcl1*$^{\Delta Myelo}$ mutation can successfully be used to study the role of neutrophils in various normal and pathological *in vivo* mouse models.
2. The neutrophil-specific deletion (*Mcl1*$^{\Delta PMN}$ mutation) of Mcl-1 anti-apoptotic protein decreased the overall number of neutrophil granulocytes to a large extent. Meanwhile other leukocyte populations were not affected in the *Mcl1*$^{\Delta PMN}$ mice compared to WT animals. However, *Mcl1*$^{\Delta PMN}$ mice showed increased mortality, decreased productivity and a poor overall phenotype compared to their WT littermates.
3. The deletion of the G-CSF receptor (*Csf3r*$^{-/-}$ mice) resulted in a moderate decrease of neutrophil numbers, and there was no change in

the size of other leukocyte populations either compared to WT animals. Even this moderate neutropenia was enough to protect the *Csf3r*$^{-/-}$ mice against autoantibody-induced arthritis.

4. We successfully proved that neutrophils are important both in the sensitization and the elicitation phase of contact hypersensitivity (CHS). We further demonstrated that the lack of inflammatory response in *Mcl1*$^{\Delta Myelo}$ mice is not caused by the deletion of Lysozyme M protein itself, since the *Lyz2*$^{Cre/Cre}$ mutant mice showed similar inflammatory response as WT animals in the CHS model.

Publications

PUBLICATIONS RELEVANT TO THE DISSERTATION:

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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. IF: 4,71 (**shared co-first autorship*)

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