First Contact Initiatives Grant report

Grant recipient: Zsofia Gulyas-Onodi MD

Home institution: Department of Pharmacology and Pharmacotherapy, Semmelweis University, Budapest, Hungary

Visited institution: Institut für Prophylaxe und Epidemiologie der Kreislaufkrankheiten, Klinikum der Universität München, München, Germany

Time of visit: October 2019

I would like to thank the ESC for allowing me the opportunity to spend time in a well-respected laboratory on the cardiovascular research field. This award was used to cover my expenses related to transportation and accommodation during my visit in Munich, Germany. I also would like to thank Prof. Dr. Sabine Steffens and for her team for introducing me to the field of flow cytometry and fluorescent-activated cell sorting (FC/FACS). This visit provided a profound training on these techniques that may contribute to further my research in the field of cardiovascular immunology. Beside my technical training, I also had a great opportunity to attend the group's lab meeting, where I held a presentation on my research. It was a pleasure to receive valuable comments that has helped improving our own work.

Background

Inflammation has been implicated as a critical contributor in the pathomechanism of cardiovascular diseases such as atherosclerosis, ischemia-reperfusion injury, myocarditis or heart failure [1-3]. Anti-inflammatory strategies are widely investigated for cardiovascular diseases including tumor necrosis factor α inhibitors, methotrexate, antibodies against cytokines or their receptors (anakinra, canakinumab); however, these anti-inflammatory drugs generally did not show clear benefit in the management of cardiovascular diseases, indicating that specific inflammatory pathways may be essential for achieving therapeutic effect [4-7].

The key players of inflammation - the immune cells - either can reside or infiltrate myocardium including macrophages, lymphocytes or granulocytes [8]. Previous papers reviewed the role of these leukocytes in cardiovascular diseases e.g. ischemic injury or non-ischemic remodeling [9, 10]; it is hypothesized that leukocytes present in myocardium orchestrate myocardial repair and remodeling. However, despite the emerging number of publications in this field, there are still several questions, which need to be addressed on the phenotype, dynamics and functions of

these cells. This investigation is important to identify specific pathways either in early and later stages of CV diseases for further research on drug development.

Therefore, our major objective is to characterize and investigate immune cells in different stages of heart disease particularly in chronic heart failure and drug-induced myocardial damage.

Report

Our project plan for the characterization of immune cells in different stages of heart failure is going to last for at least 14-16 weeks due to the chronic nature of the models, so the major objective of my stay was to learn and to practice flow cytometry analysis of cardiac immune cells and fluorescent-activated cell sorting (FC/FACS). This training will help to establish these methods in our laboratory.

In the host institution I received a general training on the application of FC/FACS in cardiovascular immunology field including the details on sample preparation, measurement of blood, bone marrow, spleen and cardiac tissue samples, and data analysis. I also took part in performing experiments with close supervision for several times in order to practice and to adapt this protocol to our laboratory.

With the help of Prof. Dr. Steffens' team, I successfully identified 1) the main populations of immune cells (granulocytes, T and B lymphocytes and monocytes/macrophages) from the most important immune organs, 2) some significant subpopulation ($Ly6C^{high}$ or $Ly6C^{low}$ macrophages, regulatory T lymphocytes) and 3) cardiac leukocytes from digested heart.

By the end of my stay I have prepared our own protocols for sample preparation and FC/FACS measurements. I practiced the experiments under supervision as well, so I had great opportunities for getting answers to all my questions. I performed detailed characterization on important immunological organs (blood, bone marrow, spleen) and heart, therefore I will be able to adapt this method to our laboratory.

We will be able to characterize our models in details after performing this pilot study in Munich (see Fig.1-4 below). By FACS, the identified cardiac macrophages and other leukocytes can be separated easily for further characterization. The analysis of leukocytes including subpopulations of macrophages isolated from healthy and failing rodent hearts might help us to identify new promising drug targets in the therapy of heart failure.

References

1. Golia, E., et al., *Inflammation and cardiovascular disease: from pathogenesis to therapeutic target.* Curr Atheroscler Rep, 2014. **16**(9): p. 435.
2. Ong, S.B., et al., *Inflammation following acute myocardial infarction: Multiple players, dynamic roles, and novel therapeutic opportunities.* Pharmacol Ther, 2018. **186**: p. 73-87.
3. Riehle, C. and J. Bauersachs, *Key inflammatory mechanisms underlying heart failure.* Herz, 2019. **44**(2): p. 96-106.
4. Kadokami, T., et al., *Anti-tumor necrosis factor-alpha antibody limits heart failure in a transgenic model.* Circulation, 2001. **104**(10): p. 1094-7.
5. Ridker, P.M., et al., *Antiinflammatory Therapy with Canakinumab for Atherosclerotic Disease.* N Engl J Med, 2017. **377**(12): p. 1119-1131.
6. Ridker, P.M., et al., *Low-Dose Methotrexate for the Prevention of Atherosclerotic Events.* N Engl J Med, 2019. **380**(8): p. 752-762.
7. Anker, S.D. and A.J. Coats, *How to RECOVER from RENAISSANCE? The significance of the results of RECOVER, RENAISSANCE, RENEWAL and ATTACH.* Int J Cardiol, 2002. **86**(2-3): p. 123-30.
8. Swirski, F.K. and M. Nahrendorf, *Cardioimmunology: the immune system in cardiac homeostasis and disease.* Nat Rev Immunol, 2018. **18**(12): p. 733-744.
9. O'Rourke, S.A., A. Dunne, and M.G. Monaghan, *The Role of Macrophages in the Infarcted Myocardium: Orchestrators of ECM Remodeling.* Front Cardiovasc Med, 2019. **6**: p. 101.
10. Chen, B. and N.G. Frangogiannis, *The Role of Macrophages in Nonischemic Heart Failure.* JACC Basic Transl Sci, 2018. **3**(2): p. 245-248.

Representative images of my results from practices can be seen below.

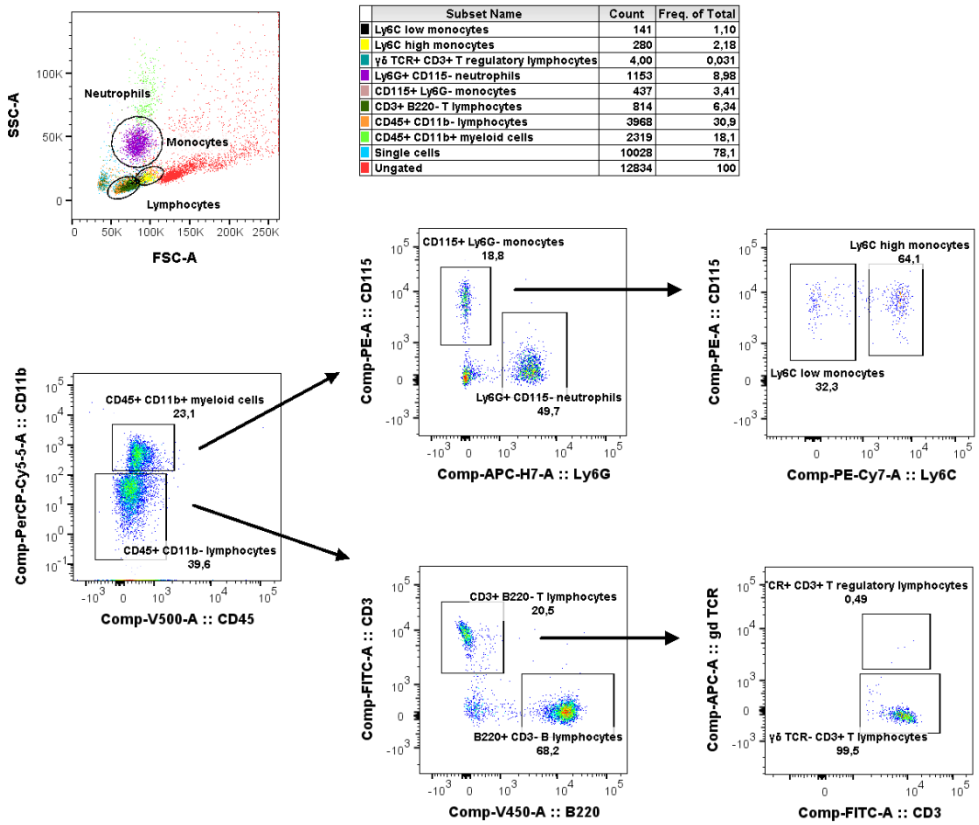
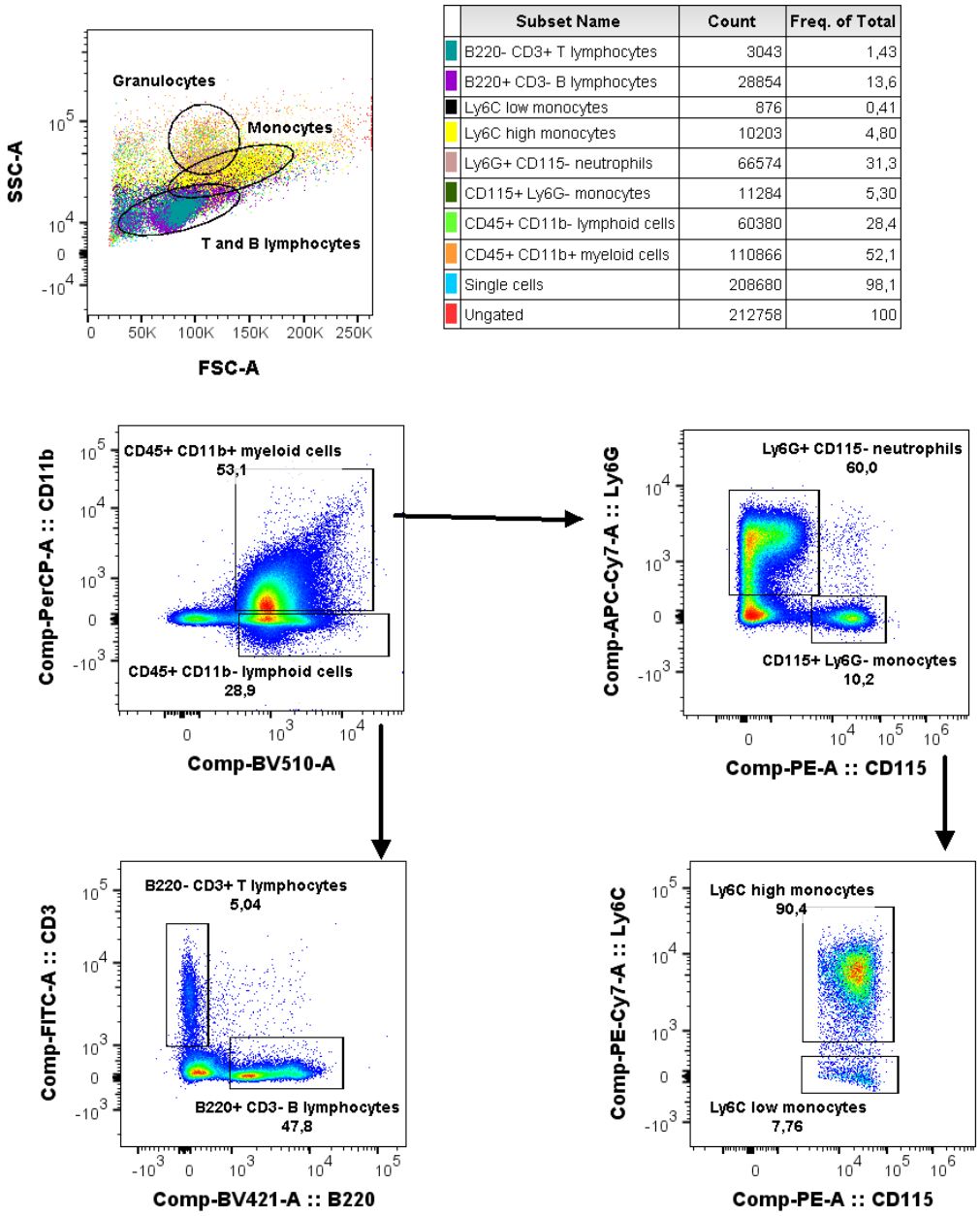
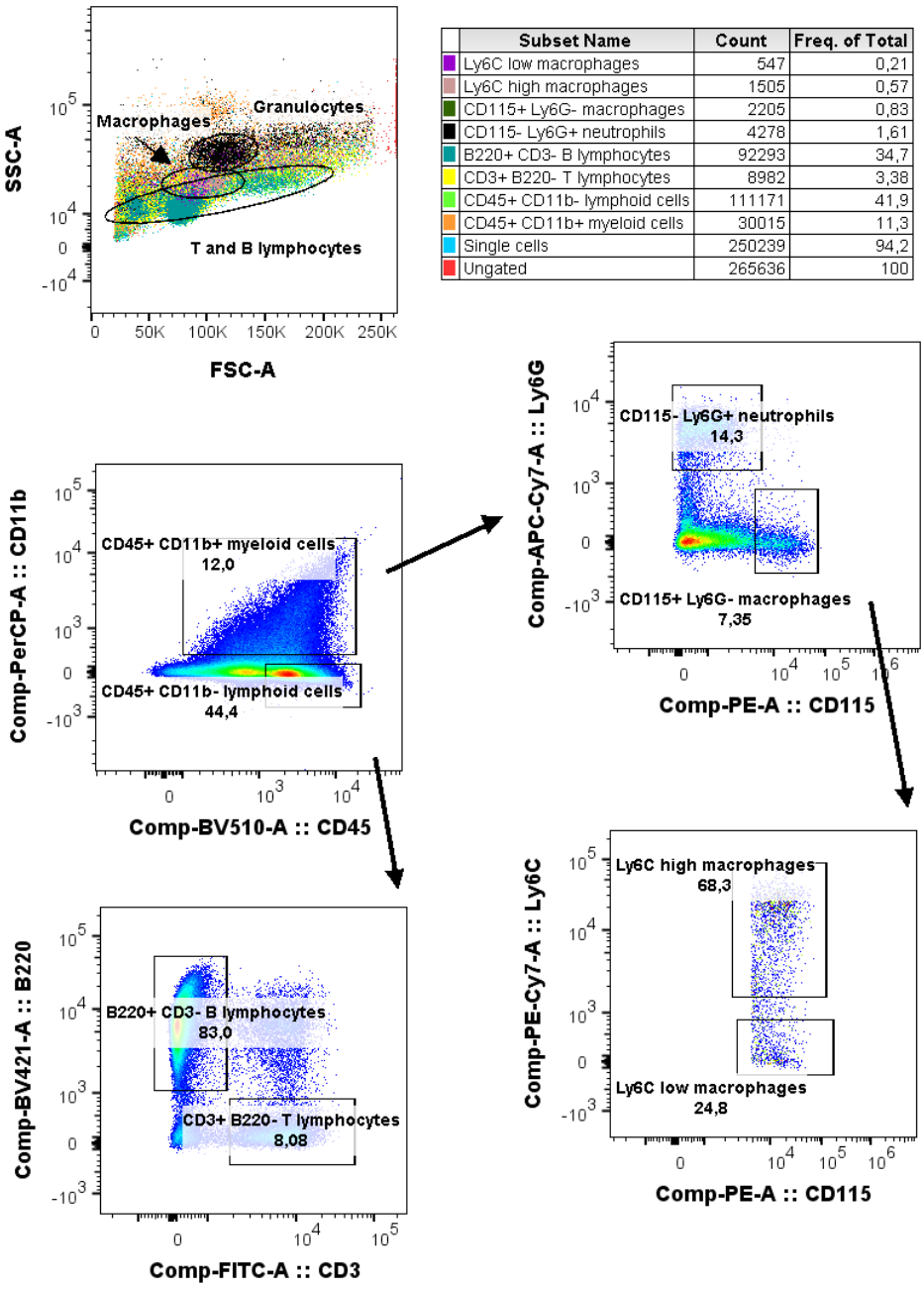


Figure 1 – Detailed characterization of circulating leukocytes in blood of a mouse by FC/FACS. The populations of neutrophil granulocytes (CD45+/CD11b+/CD115-/Ly6G+), T and B lymphocytes (CD45+/CD11b-/CD3+/B220- and CD45+/CD11b-/CD3-/B220+, respectively) and monocytes (CD45+/CD11b+/CD115+/Ly6G-) were identified. Further gating was applied to determine the subpopulations of Ly6Chigh and Ly6Clow monocytes, and regulatory T lymphocytes (δγTCR+ subpopulations). Doublets were removed prior to gating.



Subset Name	Count	Freq. of Total
B220- CD3+ T lymphocytes	3043	1,43
B220+ CD3- B lymphocytes	28854	13,6
Ly6C low monocytes	876	0,41
Ly6C high monocytes	10203	4,80
Ly6G+ CD115- neutrophils	66574	31,3
CD115+ Ly6G- monocytes	11284	5,30
CD45+ CD11b- lymphoid cells	60380	28,4
CD45+ CD11b+ myeloid cells	110866	52,1
Single cells	208680	98,1
Ungated	212758	100

Figure 2 – Detailed characterization of leukocytes in bone marrow of a mouse by FC/FACS. The populations of neutrophil granulocytes (CD45+/CD11b+/CD115-/Ly6G+), T and B lymphocytes (CD45+/CD11b-/CD3+/B220- or CD45+/CD11b-/CD3-/B220+, respectively) and monocytes (CD45+/CD11b+/CD115+/Ly6G-) were identified. Further gating was applied to determine the subpopulations of Ly6Chigh and Ly6Clow monocytes. Doublets were removed prior to gating. NOTE: CD45 was placed on axis BV510.



Subset Name	Count	Freq. of Total
Ly6C low macrophages	547	0,21
Ly6C high macrophages	1505	0,57
CD115+ Ly6G- macrophages	2205	0,83
CD115- Ly6G+ neutrophils	4278	1,61
B220+ CD3- B lymphocytes	92293	34,7
CD3+ B220- T lymphocytes	8982	3,38
CD45+ CD11b- lymphoid cells	111171	41,9
CD45+ CD11b+ myeloid cells	30015	11,3
Single cells	250239	94,2
Ungated	265636	100

Figure 3 – Characterization of leukocytes in spleen of a mouse by FC/FACS. The populations of neutrophil granulocytes (CD45+/CD11b+/CD115-/Ly6G+), T and B lymphocytes (CD45+/CD11b-/CD3+/B220- and CD45+/CD11b-/CD3-/B220+, respectively) and monocytes (CD45+/CD11b+/CD115+/Ly6G-) were identified. Further gating was applied to determine the subpopulations of Ly6Chigh and Ly6Clow monocytes. Doublets were removed prior to gating. Some overlap can be observed on CD45/CD11b panel due to compensation issues.

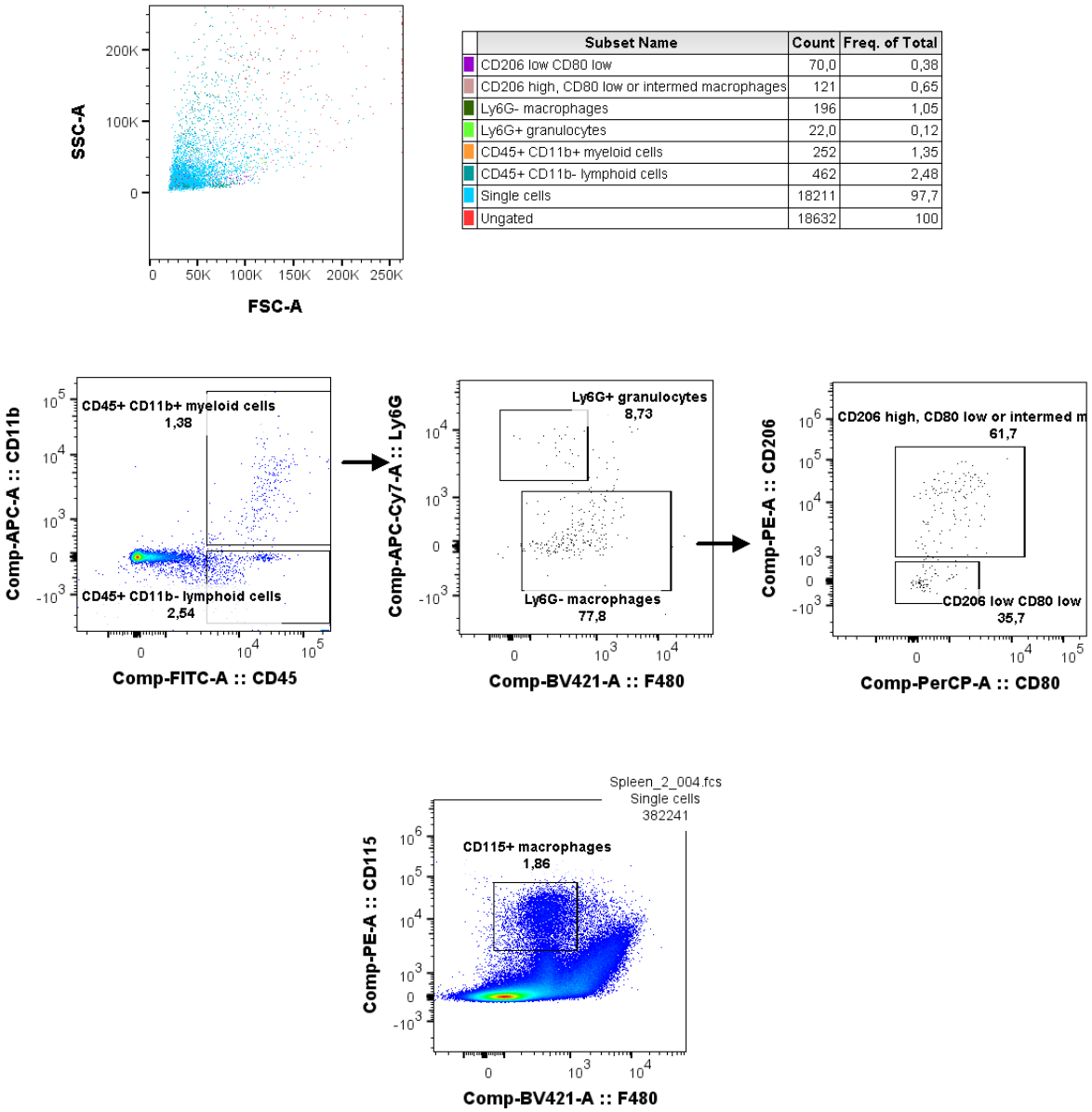


Figure 4 – Characterization of macrophages isolated from heart of healthy mouse by FC/FACS. A small number of myeloid cells (CD45+/CD11b+) and macrophages (CD45+/CD11b+/Ly6G-/F4/80int) was identified. Further gating was applied to determine the polarization of macrophages; CD80int or CD80low and CD206high or CD206low cells were identified. The low number of events and high dilution of F4/80 caused low signal (co-expression of F4/80 and CD115 was validated on spleen samples, where positive signal was appr. 102, see panel Spleen_2_004_fcs).