In vitro expansion of ex vivo regulatory T cells isolated from murine spleen and Transfection of hepatocyte growth factor (HGF) in in vitro expanded Treg for treatment of bleomycin- induced lung injury

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1. Abstract

Idiopathic pulmonary fibrosis (IPF) is a fibrotic disease of the lung with only a short median survival of three to five years after diagnosis. This short time is owed to the lack of effective therapies and current inability to stop progression of the illness due to the unknown cause. In previous experiments it has been shown that both regulatory T cells (Tregs) and hepatocyte growth factor (HGF) may slow or even stop the aggravation of the disease, if it has been applied at an early stage.

The goal of the underlying project is to test the positive effect of Tregs transfected with HGF in a mouse model, as HGF has shown to improve wound healing and may re-establish homeostasis. This necessitates protocols to ensure a stable and standardized production of these cells. Three different protocols are necessary: One for the isolation of the cells from the mouse spleen, one for the expansion and one for the transfection. To ascertain the purity and phenotype of the cells after each step, they require characterisation with flow cytometry analysis which also serves the purpose of visualizing the procedures effectiveness.

The results show that the purity of Tregs from the Stemcell Technologies isolation kit is higher than the purity obtained by the Miltenyi Biotec kit, which is the reason why it has been preferred for the expansion and further steps. The expansion shows a high amount of CD45 negative unknown cells preventing use for HGF transfection. To try and quell this population, rapamycin has been added in a further expansion experiment, yielding similar cell counts but not removing the CD45 negative population.

For future experiments, it will be important to identify these unknown cells and prevent their growth, in order to be able to continue with the transfection and eventually treat the mice.

Keywords: Idiopathic pulmonary fibrosis, regulatory T cells, Hepatocyte growth factor, expansion, transfection

2. Introduction

Idiopathic pulmonary fibrosis (IPF) is a fibrotic disease of the lung of still unknown aetiology. It has been shown that a probable cause is repeated micro-injuries of the lung epithelium which lead to dysregulated wound repair mechanisms causing a progressive loss of lung function through loss of alveolar epithelial integrity [1].

To research the cause, the mechanisms and potential therapies in the lab without endangering humans, a mouse model was established. This model is based on the administration of bleomycin into the lungs of mouse by endotracheal instillation [2]. Bleomycin has previously been used as a medication to treat cancers like Hodgkin lymphoma, non-Hodgkin lymphoma, testicular or ovarian cancer. A known side effect, amongst others, is an inflammation in the lung inducing lung scarring. This side effect is the desired effect on the mouse lung epithelium which, over the course of two weeks, will cause fibrosis. This mechanism allows study and experimentation of possible therapies of fibrosis.

As a new way to find a treatment to IPF, the goal of the lab was to transfect Tregs with HGF and then adoptively transfer them to the bleomycin induced pulmonary fibrosis mouse model. The expected effect would be to regain pulmonary immune cells homeostasis disrupted after bleomycin assault and to mitigate the progress of fibrosis. Because regulatory T cells are only present in small numbers in both blood and organs, it would be necessary to isolate and then expand them, before a sufficient amount for transfection would be available.

Flow cytometry can analyze different properties of cells. This works by first lining up the cells into a single filing, called the isoelectric focusing. The cells are then hit by one or multiple lasers, and the forward scatter, the side scatter and any fluorescence the cells show are measured. These properties help identify the cells and their subtype. To get fluorescence other than auto-fluorescence, the cells are stained with specific antibodies which are marked with a fluorescent dye.

3. Aims/ Leading Question

The main goal of the project is the establishment of protocols for cell isolation, cell expansion and HGF transfection. For this, the following questions are asked:

Which CD4+CD25+ cell isolation kit works better for the experimental design: StemCell Technologies or Miltenyi Biotec?

For the in vitro Treg expansion: Which mouse IL-2 concentration is optimal? At what time-point should the cells be harvested to obtain the optimal viability, phenotype and yield? How can the in vitro expansion affect the phenotype of isolated Tregs?

What is be the optimal HGF transfection protocol for maximal transfection rate and cell viability?

4. Material and Methods

The mice used for the isolation are BALB/c mice, as they are the most readily available in the lab. For process of the isolation, two kits and their recommended materials are used, namely the kits from Miltenyi Biotec and Stemcell Technologies. As for the protocol, the protocols of the respective kits are used.

The expansion is done with C57BL/6jRJ mice, as they are the ones used for the other parts of the corresponding study. The process of the expansion uses the Treg expansion kit from Stemcell Technologies, with an adapted protocol. Instead of the recommended 2000 U/ml of IL-2 of the kit, three different concentrations of 1000 U/ml, 2000 U/ml and 4000 U/ml of IL-2 are compared. For a later part of the experiment, rapamycin in a concentration of 100 ng/ml was added. All other material used is as recommended by the producer of the kit.

After the third expansion, a Diff Quik staining has been done with the Diff Quik kit by RAL diagnostics.

The HGF transfection is not done due to the coronavirus situation and problems in the expansion. The planned kit is the Amaxa Mouse T Cell Nucleofector Kit of the company Lonza. All other materials are the ones recommended by the producer of this kit.

5. Results

The isolations show a purity of extracted cells of around 35% for the Miltenyi Biotec kit and around 50% for the Stemcell Technologies kit. for the isolations preceding the expansions, the Stemcell Technologies kit was used.

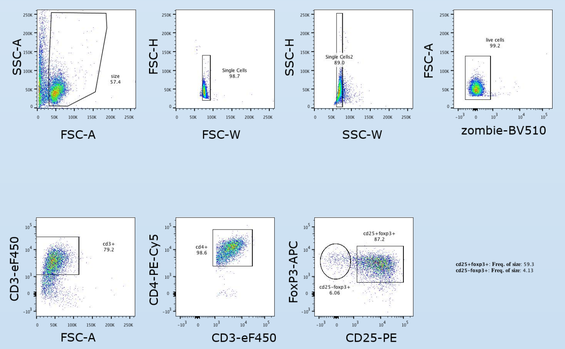

Fig. 5.1: Flow Cytometry result of the isolation with the Stemcell Technologies kit.

The expansions show a low concentration of the wanted Tregs with less than 5% of cells in all attempts being CD4, CD25, CD45 and FoxP3 positive. Instead between 85% and 95% of the cells are CD45 negative. In the third expansion, 100 ng/ml rapamycin is added, with the results showing the same percentages.

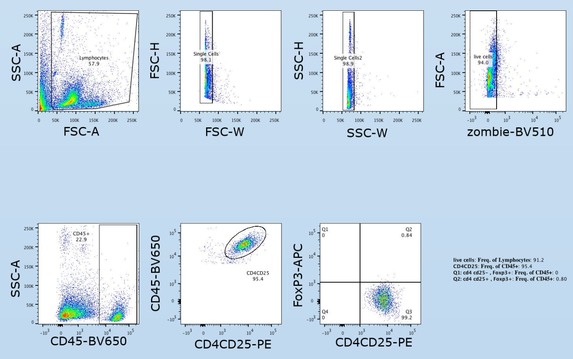

Fig. 5.2: Flow Cytometry result of the first expansion with 2000 U/ml IL-2 and without Rapamycin. A large population of CD45 negative unknown cells are visible.

The Diff Quik staining shows many dead cells, with very few living Lymphocytes.

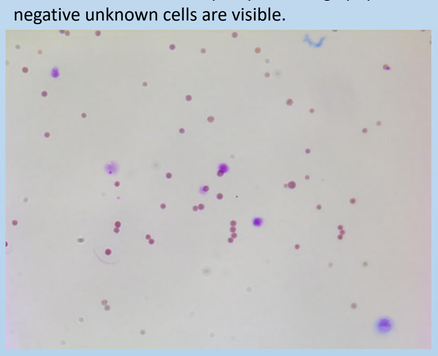

Fig. 5.3: Diff Quik Staining of expanded cells, many dead cells can be seen.

6. Discussion

The purity of the isolation is usable for the further steps, although with 50% there may still be a better method.

The purity of the expansions are not sufficient for HGF transfection. Adding rapamycin does not change the amount of CD45 positive cells and did not increase the purity of Tregs.

The Diff Quik staining shows many dead cells, which are most likely the CD45 negative cells. Altough further identification is not possible, it is necessery to prevent this population from occuring to be able to use the cells in the HGF transfection.

For the above reason and the coronavirus situation, HGF tranfection was not performed.

Further experiments may be using a different type of mouse and a different isolation method, to circumvent the problem in the expansion.

References

[1] Wolters, P.J.,Collard, H.R., & Jones, K.D. (2014). Pathogenesis of idiopathic pulmonary fibrosis, *Annu. Rev. Pathol. Mech. Dis*., 2014(9), 157-179. doi:10.1146/annurev-pathol-012513-104706.
[2] Birjandi, S.Z., Palchevskiy, V., Xue, Y.Y., Nunez, S., Kern, R., Weigt, S.S., Lynch III, J.P., Chatila, T.A., & Belperio, J.A. (2016). CD4+CD25hi Foxp3+ Cells Exacerbate Bleomycin-Induced Pulmonary Fibrosis, *Am J Pathol*, 186(8), 2008-2020. URL: http://dx.doi.org/10.1016/j.ajpath.2016.03.020.

Figures

Fig. 5.1 Herrsche, *Flow Cytometry result, Isolation with Stemcell Technologies Kit.* Bern: DBMR
Fig. 5.2 Herrsche, *Flow Cytometry result, Expansion with 2000 U/ml IL-2.* Bern: DBMR
Fig. 5.3 Herrsche, *Microscopy picture after expansion.* Bern: DBMR