Report on the Project **"Functional characterization of genetically corrected T cells from STAT3-LOF-HIES/JOB donors *in vitro* and in humanized mice"**

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1. Introduction

STAT3 (signal transducer and activator of transcription-3) is a protein from the STAT family that signals through the the Jak-Stat signaling pathway after the binding of an extracellular ligand to a cytokine receptor [1]. In this way STAT3 activation, leads to a wide variety of changes in immune cells, specifically on the differentiation of naïve CD4+ T cells to the so called T helper (Th) 17 cells which contribute significantly to the recruitment of effector cells to sites of inflammation caused by bacterial and fungal pathogens [2-3].
In humans, dominant-negative mutations in STAT3 cause hyper-IgE syndrome (HIES), also termed Job syndrome, or STAT3-loss-of-function-HIES, a primary immunodeficiency characterized by the susceptibility to bacterial and fungal infections, as a consequence of a reduced numbers in Th17 cells [4-6].
Current treatment of these patients is entirely symptomatic and antibiotic and fungal prophylaxis is given life-long. In order to improve the infectious burden and the quality of life of the patients, it has been suggested to carry out a hematopoietic stem cell transplantation (HSCT) in STAT3-LOF/HIES/Job patients. However, when performing an HSCT in adults, we fear that the outcome will not be as favorable, as pre-existing lung conditions and the infectious burden (including but not limited to cytomegalovirus, adenoviruses, and aspergillus) will complicate the procedure. To address this problem, Prof. Cathomen (co-applicant) has developed a procedure to genetically correct HIES patients T cells in order to give the genetically corrected cells (after having expanded them *in vitro*) back to the patient.
The initial step in this project was to evaluate the capacity of those genetically corrected T cells to differentiate into the Th17 cell subtype, and to establish a humanized mouse model, in which the injection of genetically corrected T cells into these immunodeficient mice, will allow us to evaluate the survival and differentiation capacity of those injected genetically corrected T cells *in vivo*.

We present here the results obtained during the year 2022.

2. Results

2.1 Th17 *in vitro* differentiation of healthy naïve T cells

The *in vitro* differentiation of naïve CD4+ T cells into Th17 cells was performed in order to establish the protocol to later differentiate the edited STAT3 naïve T cells from our co-applicant Prof. Dr. Tony Cathomen. The differentiation started by FACS sorting CD4 naïve T cells ($CD4^{+}CD25^{-}CD45RA^{+}CCR7^{+}$) from healthy donor (HD) peripheral blood mononuclear cells (PBMCs) (Fig. 1).

After sorting, cells were cultured in anti-CD3 antibody coated plates, with soluble anti-CD28 antibody in order to induce their activation, together with a cytokine cocktail (IL-1, IL-6, IL-23) that induces their differentiation towards Th17 lineage. Cells were cultured for 7 days, and the last 5 hours, cells were stimulated with Phorbol 12-myristate 13-acetate (PMA) together with the calcium ionophore, Ionomycin, to induce the secretion of the cytokine IL-17, as a read out of their final differentiation. We could determine an increase in the IL-17 producing cells after culturing cells with IL-1+IL-6 and with IL1+IL-23 (Fig. 2), and this differentiation was induced at day 7 of the culture, since at day 1 we could not detect any IL-17 producing cells, confirming that our initial starting material corresponded to fully naïve CD4+ T cells.

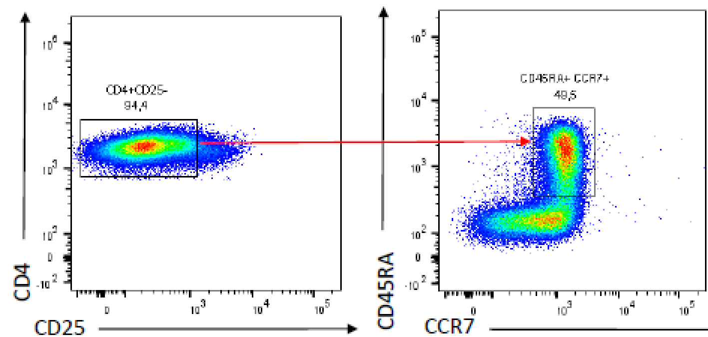

Figure 1. Healthy donor Naïve CD4+ T cells were FACS sorted as $CD4^+CD25^-CD45RA^+CCR7^+$ from total PBMCs.

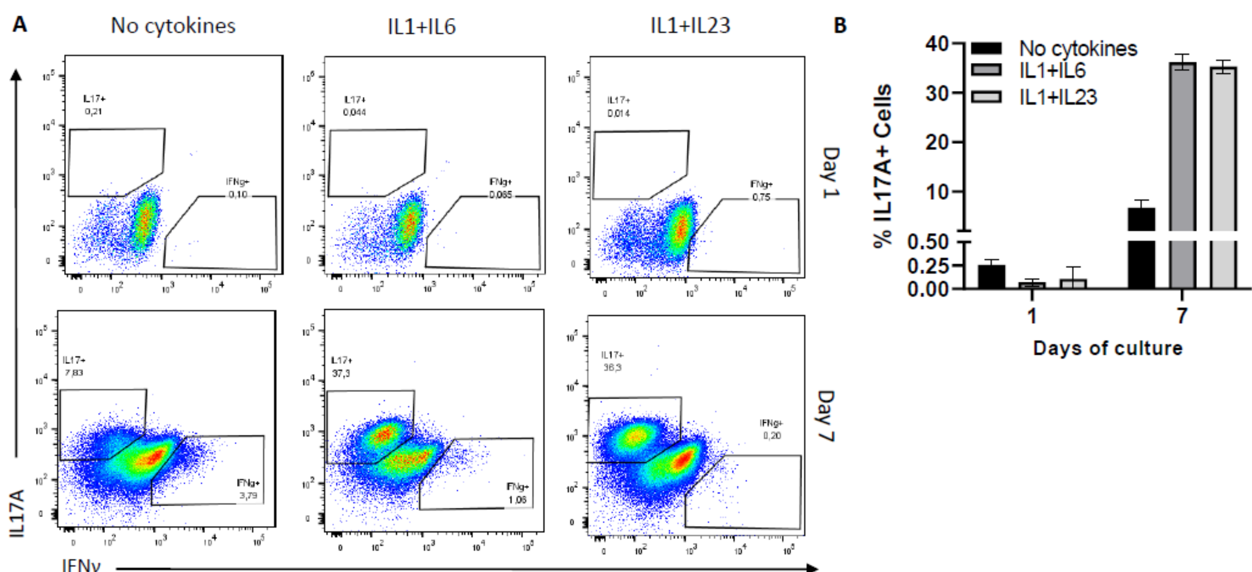

Figure 2. (A) Healthy donor Naïve CD4+ T cells were cultured in the absence (No cytokines) (left) or in the presence of the cytokine combinations IL1+IL6 (middle) or IL1+IL23 (right) for 1 day (upper panels) and for 7 days (lower panel). Before analyzing the secretion of IL17A and IFNγ by Flow Cytometry, cells were stimulated with PMA + Ionomycin + Brefeldin A for 4 hours. (B) Mean (±SD) frequencies of IL17A+ cells as gated in *A*.

2.2 Characterization of edited STAT3 cells.

The protocol of the edited STAT3 cells from the laboratory of Prof. Cathomen, our co-applicant, included a three-day activation with anti-CD2, anti-CD3 and anti-CD28 antibodies of total PBMCs, followed by the nucleofection step with the corrected STAT3 gene, and finally another 2 weeks of expansion, after which we were expecting to differentiate those cells into Th17. During this time T cells may have changed their phenotype and characteristics, and possibly also their capacity to become Th17 cells. Therefore, we decided to first characterize the cells by surface marker expression, in order to better define them as naïve CD4+ T cells. To this end, we included several surface markers (CD3, CD4, CD45RA, CD45RO, CD62L, CCR7) and we were able to document that these cells have a completely different phenotype than the phenotype observed in naïve CD4+ T cells.

As can be seen in Fig. 3A, there is a high percentage (61%) of cells that are CCR7-, indicating their non-naive status, as well as a high percentage of cells (79,8%) expressing the T cell memory marker CD45RO (Fig. 3B). This data indicates that the cells are showing a different phenotype to the classical naïve CD4+ T cells, which could influence in their differentiation capacity. Nevertheless, we continued with our experiment to differentiate these cells towards the Th17 cell lineage (see below point 2.3).

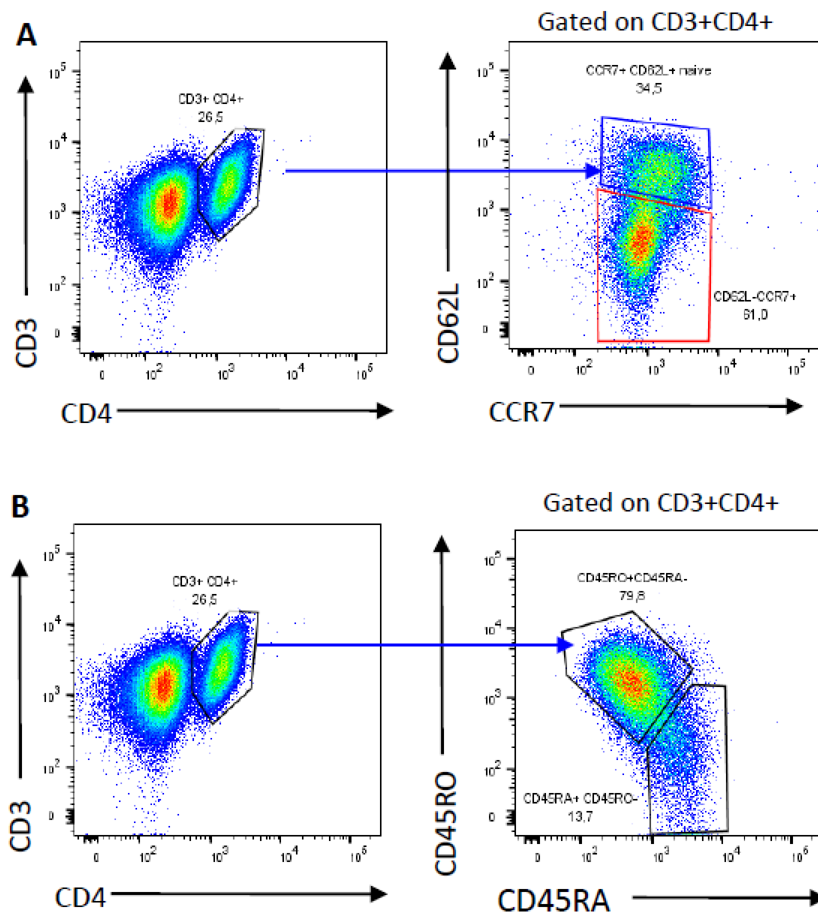

Figure 3. HD cells were cultured for two weeks after nucleofection with specific vectors (Prof. Dr. Toni Cathomen) and the staining for the different naïve T cell markers was analyzed by Flow Cytometry. Cells were considered Naïve T cells as $CD3^+CD4^+CD62L^+CCR7^+$ (A) and $CD3^+CD4^+CD45RO^-CD45RA^+$ (B).

2.3 Differentiation of mock-nucleofected corrected T cells

The fact that the cells changed their phenotype after two weeks of expansion, is an important point to consider when initiating the Th17 cell differentiation culture. Considering this, we took different approaches to achieve our Th17 cell differentiation culture, using mock-nucleofected cells as a way to set-up our differentiation protocol.

After cells were mock-nucleofected, we divided them in two groups, one group rested overnight (ON), and the second group stayed in culture for two weeks, in order to mimic the protocol of nucleofection, in which cells are expanded for two weeks. To both of those groups, cells were either not activated or activated with anti-CD3 and anti-CD28 antibodies, because we wanted to evaluate whether this activation process would also affect their differentiation. Cells were kept in culture for 7 days and the last day, the supernatant of the cell cultures was taken to measure by ELISA the IL-17 levels.
In this experiment we also included cells from a Hyper-IgE patient as a negative control for the differentiation.
Fig. 4 shows a representation of the above described protocol.

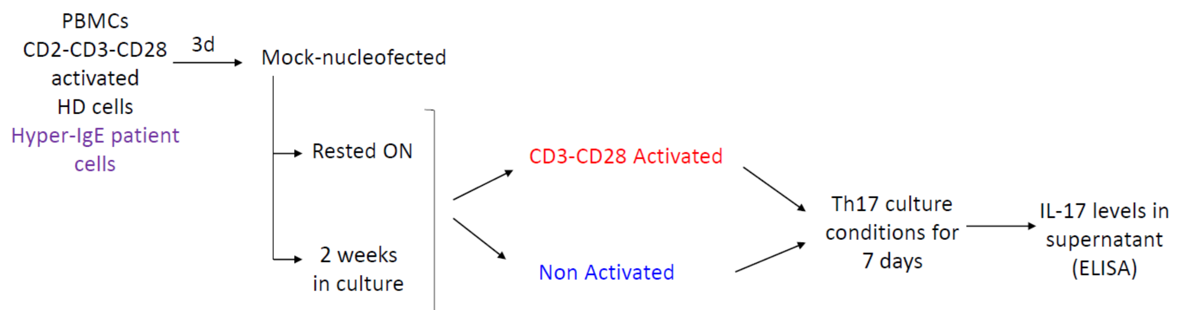

Figure 4. Schematic representation of the protocol used for the differentiation of the mock-nucleofected cells.

As shown in Fig. 5A, we could observe that when cells were rested overnight, we detected IL-17 in the supernatant of HD cells culture with IL-1+IL-6 and with IL-1+IL-23, while no detectable levels of IL-17 were observed in the supernatant derived from Hyper-IgE patients´ cells.
A similar result was observed when cells were rested for two weeks (Fig. 6A), but in this case IL-17 levels were significantly increased in HD cell cultures, comparted to the patients' cells, only when cells were cultured with IL-1+IL-6.
Notably, the IL-17 secretion was only detected when cells were activated with anti-CD3 and anti-CD28 antibody (compare Fig. 5A with 5B and Fig. 6A with 6B), indicating the importance of the T cell activation for the Th17 differentiation process.

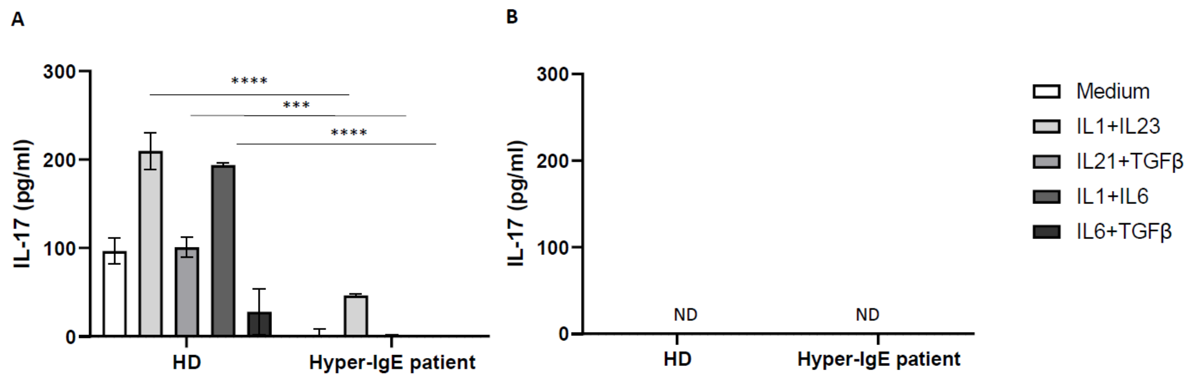

Figure 5. IL-17 levels in supernatants from Healthy Donor (HD) and Hyper-IgE patient cells, cultured for 7 days under the presence of different cytokines. After mock-nucleofection, cells were rested ON and activated (A) or not (B) with anti-CD3 and anti-CD28 antibodies. ND: not detected. *** p< 0,001; **** p< 0,0001

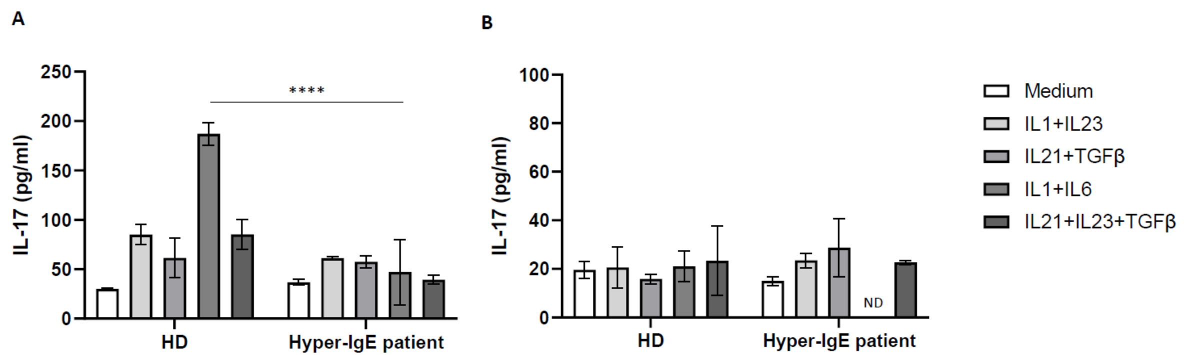

Figure 6. IL-17 levels in supernatants from Healthy Donor (HD) and Hyper-IgE patient cells, cultured for 7 days under the presence of different cytokines. After mock-nucleofection, cells were culture for 2 weeks and activated (A) or not (B) with anti-CD3 and anti-CD28 antibodies. ND: not detected. **** p< 0,0001

These results indicate that, even the cells after nucleofection show a different phenotype to the one of a naïve CD4+ T cells, they still have the capacity to differentiate to Th17 cells.

2.4 Set up of a humanized mouse model

The remaining goals of this project were associated to establishing a humanize mouse model. We have been working on the Animal Project Proposal that is needed to be approved by the regulatory officials of the City of Freiburg, which it was successfully presented on December 2022 (Project # 35-9185.81/G-22/114). We expect that the evaluation and the final approval of our Animal Project Proposal from the government of Freiburg, will take approximately 3 months.

The initial proposal was designed by injecting edited T cells to NOD-SCID-Gamma or “NSG” mouse (NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ), which are defective in innate and adaptive immune functions due to the lack of innate immune cells as well as T and B cells [7]. Taking into account that our collaborators are working also on editing hematopoietic stem and progenitor cells (HSPCs), one additional possibility that we will have is injecting mice with HSPCs in addition to T cells. This should not be a problem, since the HSPCs injection in immunodeficient mice is a successful established model for the creation of a humanized mouse model [8]. Therefore, we are confident that we will succeed on setting up this model, which is necessary for the evaluation of the restored STAT3 function on the edited cells.

3. Summary and Outlook

During the first year-and a half of our project, we could establish the Th17 differentiation protocol for HD naïve CD4+ T cells that allowed us to define the conditions for further differentiation experiments, specifically for those involved with the edited STAT3 cells from our co-applicant. Furthermore, we were able to characterize those cells by FACS and we could determine that they have a different phenotype to the one of naïve CD4+ T cells, as a consequence of their expansion which is necessary for the protocol of editing STAT3. Nevertheless, we were able to detect IL-17 cytokine levels in the supernatant of cell cultures derived from HD cells that were either rested overnight or for two weeks under activation conditions, compared to almost undetectable levels in supernatants derived from Hyper-IgE patient’s cells.

One of the most important goals of this project is the development of a humanized mouse model. We are currently waiting for its approval from the government of Freiburg which can be considered as one important delay on this project. Despite that, we are confident that the establishment of this model will give us clear information about the *in vivo* functionality (survival rate, differentiation capacity and response to infection) of the edited STAT3 cells.

4. Layman Summary

Hyper-IgE syndrome is caused by different mutations in STAT3, an important factor for the development of a so called T helper (Th) 17 cell, relevant for the response against bacterial and fungal infections. In this project, our goal is to reach this differentiation *in vitro*, from healthy non differentiated cells, named naïve cells, but also from cells derived from patients suffering from Hyper-IgE syndrome that were previously corrected to have the normal STAT3 gene.

In this project, we could demonstrate that we were able to differentiate healthy naive cells to Th17 cells *in vitro*, and we could show that the process of correcting the STAT3 gene in the cells, changes their phenotype and probably, their capacity to become a Th17 cell. However, we could manage to detect the cytokine IL-17 in the medium where those cells were cultured, with specific activation signals and other differentiating factors.

Moreover, these results are important for the main goal of this project that is using these STAT3 corrected cells for creating a mouse model, in which we can evaluate the functionality of those cells, in particular, their capacity to survive and to respond to an infection.

The results obtained in these future experiments will be relevant for establishing novel future therapeutic treatments to patients with Hyper-IgE syndrome.

5. Acknowledgements

We thank to our collaborator and co-applicant, Prof. Dr Tony Cathomen, for providing the cells to optimize our differentiation protocol and for fruitful discussions, as well as to the patients who donated blood samples for our research, and finally to the Job Research foundation for financing this project.

6. References

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