Understanding Severe Allergic Phenotypes: Immuno-energetic Phenotyping of Olive-Allergic Patients Treated with Dupilumab

Type of fellowship: Medium-Term Research Fellowship

Home Institution: San Pablo-CEU Universities (Madrid, Spain)

Host Supervisor: PD. Dr. Dr. Med Milena Sokolowska

Host Institution: Swiss Institute of Allergy and Asthma Research (SIAF) (Davos, Switzerland)

Duration: 6 months (February 1st – July 31st)

1. Background

Allergy is one of the most prevalent chronic diseases worldwide, with around 10% of allergic individuals developing a **severe phenotype** of the disease, which is accompanied by different clinical manifestations (e.g. asthma, polyposis, rhinitis...), a reduction in their quality of life - associated with an increase in the number of exacerbations and hospitalizations – and a high economic impact on their healthcare systems[1–3]. To confront this complexity, a deeper understanding of the molecular and cellular mechanisms underlying the development of severe allergic phenotypes became essential, aiming to progress in the generation of more effective and personalized therapeutic approaches.

In general, **an allergic reaction is characterised by an exacerbated inflammatory response of T helper 2 cells (Th2)** that may be perpetuated, in part, by impaired control of other cells with a potent immunoregulatory role such as regulatory T cells (Treg)[4–7]. Specifically, Treg cells can mediate the response of Th2 cells to allergens through different mechanisms such as the release of inhibitory cytokines (IL10, TGF-β), modulation of dendritic cell functions or deprivation of trophic cytokines (IL2)[6,8–10], and the loss of their regulatory function is associated with the development of uncontrolled asthma[11]. Indeed, some individuals with a severe allergic phenotype do not respond to conventional treatments (e.g. inhaled corticosteroids, bronchodilators, allergen-specific immunotherapy (AIT))[1,12], needing add-on treatments.

This project focused on **olive tree (*Olea europaea*) pollinosis**, a major cause of respiratory allergy in the Mediterranean area[13]. Among the 14 allergenic proteins, Ole e 1 is the most abundant sensitising agent, however, in regions with extreme olive pollen exposure (e.g. Cordoba, Andalusia, Spain), other minor allergens such as **Ole e 7** become dominant in the sensitisation profile of the allergic patients[13]. Ole e 7-positive patients manifest a severe allergic phenotype characterized by increased numbers of Th2 and innate lymphoid type 2 cells (ILC2s), as well as decreased numbers of Tregs, usually accompanied by elevated levels of the Th2/Th1 cytokine ratio[14–16]. This cellular and molecular phenotype suggests that Ole e 7-sensitised

patients show a consistent dysregulation of the immune response associated with T cell function. This may be related to the poor response or systemic allergic reactions to AIT and the development of severe allergic phenotypes[17–22].

In the last decade, **new biological agents** have been designed to intervene in the aberrant Th2 signalling response present in severe allergic patients by blocking different molecules (e.g. IgE, IL-5, IL-4 or IL-13), in most forms of asthma, polyposis and/or atopic dermatitis[22–26]. However, although clinical benefits have been proven, little is known about the systemic immunometabolic consequences of blocking the Th2 response[27]. In this project, we will be focused on **Dupilumab - *Dupixent*®**, a monoclonal antibody that simultaneously blocks the IL-4 and IL-13 signalling pathways through direct interaction with the heterodimeric IL4Rα/IL-13Rα1 receptor[28,29]. Dupilumab has demonstrated strong clinical efficacy in different allergic phenotypes such as atopic dermatitis and asthma[30–32]. We, therefore, propose that Dupilumab treatment of Ole e 7-sensitised patients could help restore immune homeostasis in these severe phenotypes.

2. Aim and Objectives

This project aims to evaluate the effect of Dupilumab at the cellular and molecular level in 22 patients sensitised to Ole e 7 with severe asthma (steps 4-5 of the GINA guidelines[1]), who have not responded to standard treatment (e.g. corticosteroids, short/long-acting β2 agonists).

To this end, given that the home laboratory is currently working with different **omics techniques** (genomics, proteomics, metabolomics), the samples of this project will be analysed by metabolomics and proteomics in the home laboratory. However, to complement this molecular characterization, we will perform a cellular characterization in the host laboratory by analysing the **functional and immunometabolic changes** that may occur in the different immune populations (different subtypes of T cells (Th2, Th9, Treg) and ILCs (ILC1, ILC2, ILC3)).

Accordingly, the **main objectives** of this internship are:

1) To acquire experience in the use of multichannel flow cytometry, and to carry out the immunophenotyping of different immune subpopulations of the samples. Likewise, to characterise the bioenergetic requirements of these cells by employing the SCENITH technique, which have been previously optimised and used in different studies carried out in Dr Sokolowska's group at SIAF.

2) To use these techniques on the immunophenotyping of the Peripheral Blood Mononuclear Cells (PBMCs) samples obtained from Ole e 7 allergic patients treated with and without Dupilumab treatment.

3) To relate specific changes in cellular immuno-profiling, before and after Dupilumab intervention, with their bioenergetic requirements.

3. Adaptations from the research plan

At the beginning of the research stay and just a few days before shipping the PBMCs samples from the patients, there was a problem with the storage conditions of the samples. The PBMCs were exposed to higher temperatures than recommended for an extended period. As a result, cell viability was checked (**Figure 1**) and it was determined that the samples were no longer suitable for the planned analyses.

To move forward with the project, we decided to apply the same experimental approach (combining flow cytometry with the SCENITH technique) to an already available cohort of patients allergic to grass and/or birch pollen, who had been treated with subcutaneous AIT for three months. For these patients, PBMCs were collected at four time points: before treatment, and at 3, 6, and 12 months after completing it. Samples from healthy donors were also included.

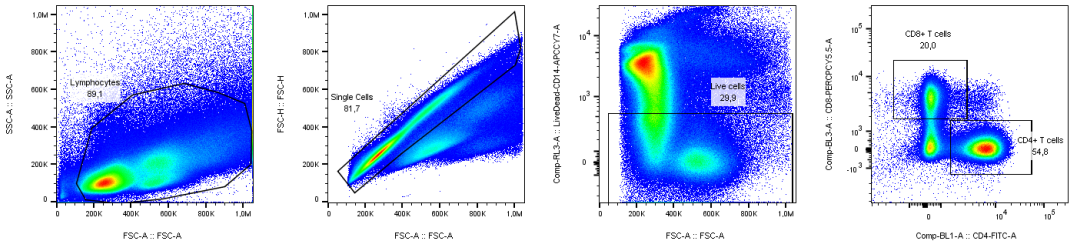

Figure 1. Flow cytometry analysis of the viability of the PBMCs obtained from the Ole e7-sensitive patients. A 30% of viability was shown in the third gating.

This cohort had already been partially studied to evaluate trained immunity and innate cell responses[33], showing that AIT can modulate the composition and function of innate immune populations. Building on that, the aim of the new study is to expand the analysis to T cell and ILC subsets, in order to better understand their functional and metabolic profiles after AIT, an area where knowledge is still limited[34].

4. Methodology and Results

The optimization of the protocol was performed using PBMCs from healthy donors. We started with $20x10^6$ PBMCs, which matches the number of cells collected from the patients enrolled in the project.

4.1. Optimization of the flow cytometry panel

To perform immunophenotyping and assess the functional profile of patient's immune cells, we designed a multicolour flow cytometry panel to identify conventional and pathogenic Th2 cells, regulatory T cells and ILC subsets (**Figure 2**).

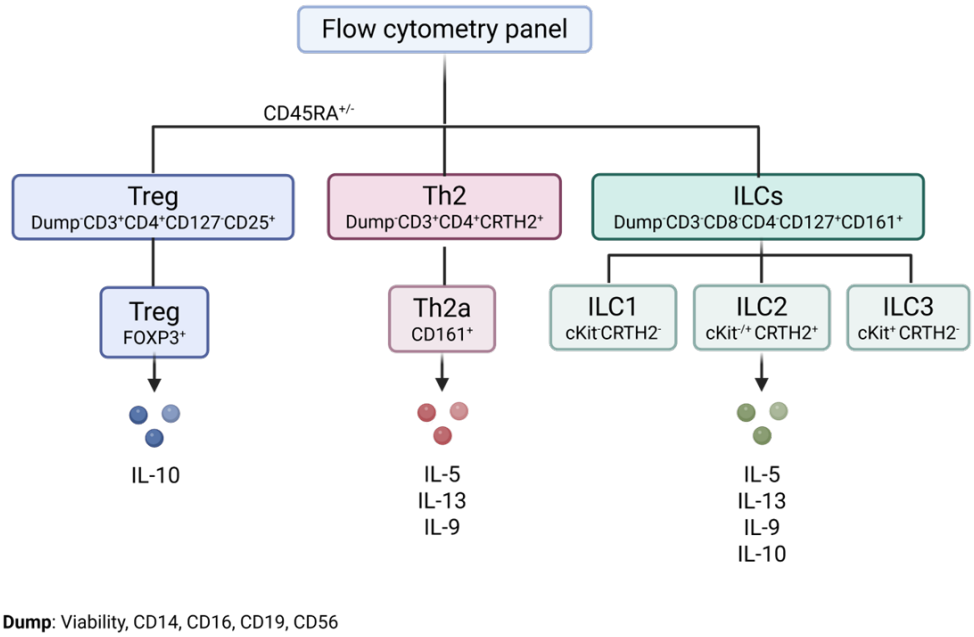

Figure 2. Diagram showing the different markers including the multicolour flow cytometry panel to perform the immunophenotyping and determine the functional profile of the different immune subsets analysed.

Each marker in the panel was titrated individually to ensure optimal staining and accurate identification of all targeted immune cell populations. For intracellular cytokine detection, cells were first stained for the surface markers, then fixed and permeabilized for intracellular staining. The panel was specifically optimized for use on a BD LSRFortessa flow cytometer (BD Biosciences).

4.2. Optimization of the stimulation conditions

Once the panel was established, we proceed to optimize the stimulation conditions needed to assess the functional profile of the different immune cell populations. We looked for a method in the literature that would activate both T cells and ILCs effectively.

PMA and Ionomycin are commonly used to stimulate these cell types, but different studies have used different concentrations. To determine the best condition for our experiment, we tested six different combinations. In addition, Brefeldin A was also included in our conditions, since it is known to block protein transport form the endoplasmic reticulum to the Golgi improving intracellular cytokine detection (**Table 1**).

After testing all six conditions, condition 3 was selected as it resulted in the highest detection of IL-5, IL-9, IL-13 and IL-10 positive cells.

Table 1. Conditions for T cell and ILCs stimulation.

Condition	PMA (ng/mL)	Ionomycin (µg/mL)	Brefeldin A (µg/mL)	Time (hours)
1	10	0.5	10	4
2	10	1	1	4
3	25	1	10	4
4	50	1	-	4
5	-	-	10	4
6	25	1	10	2+2

4.3. Optimization of SCENITH technique

To analyse cellular metabolism, we performed SCENITH, which aims to analyse the energy metabolism of immune cells using protein synthesis as a readout of total metabolic activity[35]. Starting with $20x10^6$ PBMCs, cells were first divided into two main conditions based on stimulation:

- **Stimulated cells**: incubated for 4 hours in complete medium (cRPMI) with 25 ng/mL PMA, 1 µg/mL Ionomycin, and 10 µg/mL Brefeldin A.
- **Unstimulated cells**: incubated for 4 hours in cRPMI only.

After incubation, we proceeded with the SCENITH protocol. Each of the two initial conditions was split into five metabolic conditions:

- **PBMCs + DMSO**, as a negative inhibition control.
- **PBMCs + 2-deoxyglucose (2-DG)**, as a glycolysis inhibitor.
- **PBMCs + oligomycin**, as an inhibitor of oxidative phosphorylation (OXPHOS).
- **PBMCs + 2-DG + oligomycin**, to block both glycolysis and OXPHOS.
- **PBMCs + harringtonine**, as a positive control for blocking protein translation.

Cells were treated with the respective inhibitors for 15 minutes, followed by 30 minutes incubation with puromycin, a tyrosyl-tRNA analogue that blocks protein translation. This allowed us to assess protein synthesis, and indirectly, the bioenergetic status of the cells by flow cytometry using a fluorescent anti-puromycin antibody.

After the puromycin incubation, we performed full immunophenotyping of the specific immune populations (i.e., Th2, Th2a, Treg, ILCs (ILC1, ILC2, ILC3)). For this, cells were incubated for 30 minutes at 4°C with surface markers (i.e., Viability, CD14, CD16, CD19, CD56, CD4, CD8, CD3, CD45RA, CD25, CD127, CD161, CRTH2). We then fixed and permeabilized the PBMCs for intracellular staining using selected markers (i.e., puromycin, FOXP3, IL-5, IL-9, IL-10, IL-13), with an hour of incubation at room temperature.

Finally, samples were acquired on a BD LSRFortessa flow cytometer. Data were analysed using FlowJo™ v10.0 to characterize the samples at the population, functional, and metabolic levels.

5. Work program and results

During these six-month mobility period, the work program:

TIMETABLE	February				March				April			
	W1	W2	W3	W4	W1	W2	W3	W4	W1	W2	W3	W4
Basic laboratory training and familiarisation with the flow cytometer in the	X	X	X									
Gain experience with flow cytometry methodology		X	X	X	X							
Fine-tuning of the antibody panel developed for immunophenotyping					X	X	X	X	X	X	X	X
	May				June				July			
	W1	W2	W3	W4	W1	W2	W3	W4	W1	W2	W3	W4
Fine-tuning stimulation assay	X	X	X	X	X							
Fine-tuning of SCENITH technique						X	X	X	X	X	X	X

By the end of the mobility period, the optimization of the entire protocol was completed, and the protocol is now ready to be applied for the analysis of the patients' samples. Finally, data analysis of the obtained results will be conducted at the home lab in collaboration with the host institution with remote support provided to complete the study.

6. Acknowledgements and personal reflection

I would like to begin expressing my gratitude to Dr. Milena Sokolowska for supporting my application and hosting me over the past 6 months in her Immune Metabolism group at the Swiss Institute of Allergy and Asthma Research (SIAF). I am deeply thank you for everything I have learned from her and the whole team. I also want to express my gratitude to Abhi, whose support has been truly remarkable. Thank you to all SIAF people for the warm welcome and for making my stay much easier.

During this research stay, I had the opportunity to gain experience in flow cytometry and immunometabolism. I also attended weekly SIAF seminars and group meetings, which provided a broader learning. These experiences have been instrumental in my growth as a scientist and have allowed me to build meaningful relationships with my host institution and its members.

Lastly, I would like to express my sincere thanks to EAACI for awarding me this Medium-Term Research Fellowship and for making this enriching experience possible. This fellowship enabled me to collaborate with exceptional scientists in the field of allergy and immunometabolism.

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