Final Report

Mid Term Research Fellowship

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Host Supervisor: Cezmi Akdis, MD, Prof. Dr. med.

Co-host supervisors: Milena Sokolowska, MD, PhD.

Location: Swiss Institute of Allergy and Research (SIAF), Davos, Switzerland.

Research title: "Immunophenotyping, transcriptomic and metabolomics of PMBCs from olive allergic patients"

This report describes my research project, activities and achievements over the period of my fellowship (1 October 2018-31st March 2019) at the Swiss Institute for Allergy and Asthma Research (SIAF), Davos, Switzerland.

BACKGROUND AND RATIONALE

Allergy is a chronic disease in which an allergen incites an extreme response for the immune system. Repetitive stimulus by an allergen tends to worse the disease and sensitization to new epitopes/allergens (1, 2). During the last decades the prevalence of allergy has increased in industrialized and developing countries(3). New interventions have been developed to control the symptoms of the disease during prolonged of time such as allergen-specific immunotherapy (AIT) (4, 5). This is based on delivering low, increasing amounts of the allergen to the patients to build immunotolerance (6-8).

Olive pollen is one of the major aeroallergens present in the south of Spain. Patients usually develop asthma, but in the worst scenario, they react very strongly against minor allergens such as Ole e7 and Ole e9. The treatment by AIT usually induces a long-time remission(9), prevents sensitization to new allergens (10-12) and asthma development(13). However, in those severe cases the treatment is not well tolerated as it induces to these patients a systemic allergic reaction(13). Not only is that, but sensitization to Ole e7 has been associated to plant derived food anaphylaxis(14). Therefore, these patients constitute a unique model of great pathophysiological interest as they represent a big problem for the health care system, as it is a chronic disease without a specific and safe treatment.

We hypothesize that there is a specific cell-based and/or metabolite-based factor that drives these systemic allergic responses after AIT. These potential causative metabolites in the serum, could also originate from the gut microbiota. These differences could be detected by novel approaches of immunophenotyping, lipidomics, and metabolomics of the T cells, ILCs and serum of these patients. One of the possible factors that could influence AIT failure could be the variability in the relative content of minor allergens (15). Nevertheless, recently it has been found that patients with similar IgE sensitization profiles present different response to treatment with the same AIT extract (16). Consequently, certain cell populations may be pre-conditioned to elicit a systemic response after AIT.

Furthermore, our previous results in a similar population of patients pointed out that in serum of severe allergic patients there is a depletion of arachidonic acid precursors with concomitant elevation of sphingosine1-p production as well as Warburg carbohydrate metabolism (17).

Warburg metabolism, also called aerobic glycolysis, is described as the use of glycolysis as primary source of ATP anabolism in presence of oxygen. Aerobic glycolysis has been described as the typical metabolism for activated T cells (18-20). This type of metabolism has been shown to be exceptionally important in mice to develop Th2 responses. Inhibition of glycolysis is able to block GATA3 transcription, stop Th2 differentiation and decrease the production of IL-4 (21, 22). This could be different in an established disease since Th2 cells from asthmatic individuals presented higher levels of carnitine palmitoyltransferase 1a (CPT1a) than healthy controls (23). CPT1a is known for transporting fatty acids to the mitochondria to generate ATP by β–oxidation, which could signal the importance of this metabolism pathway for Th2 in humans.

On the other hand, T regulatory cells (T regs), the main suppressors of Th2 cells in allergy (24), are believed to mainly use fatty acid and acyl compounds as the main carbon source supply to generate ATP(19, 20, 25). Instead, T regs seem to depend on glycolysis for migration purposes (26). Because of this metabolism, T regs seem to have developed the ability to incorporate lipids from the environment for the membrane, which would make them dependable of them to exert their functions (27). Having observed in our past study a decrease in xenobiotic lipids derived probably from the gut microbiota in severe allergic patients (17), we believe that it can be reflected by a change in the phenotype of T regs or their function. Subsequently, they will need to change their metabolism to prevent the collapse of their cell membrane, which would compromise their function. This could be a potential explanation to the systemic reactions after AIT of the severe allergic patients and microbial metabolites could be very relevant as they have been already associated with lung inflammation (28, 29).

Finally, we want to study the ILC compartment in the severe patients. ILCs are known to produce multiple types of pro-inflammatory and immunoregulatory cytokines (30). ILC2s seem especially important for allergic inflammation in mice as they have been shown to promote production of Th2 cytokines and airway inflammation (31). Not only that, but ILC2s have been shown to migrate via sphingosine one phosphate (S1P) chemotaxis and accumulated in the lung (32). We observed previously and increased level of S1P, which could signal mobilization, and activation of ILC2s, which could be related to the patients' symptoms (17). In obese patients as leptin seems to enhance their pro-inflammatory behavior although their concrete function in allergy is not completely understood (33). However, the exact role in allergy and the metabolism of innate lymphoid cells has not been studied in details in humans.

In summary, to gain insight in the metabolism of immune cell compartment in severe allergic patients anaphylactic during AIT we aim to: i) immunophenotype T regs, T effector cells as well as subpopulations of ILCs isolated from blood; ii) to investigate their metabolic functions.

AIM OF THE STUDY

To characterize T cells and innate lymphoid cells (ILCs) subpopulations and their metabolic activity/functionality in severe allergy to olive pollen

DETAILED OBJECTIVES

1. To perform detailed immunophenotyping of T cells and ILCs from severe allergic patients with anaphylactic reactions during allergen-specific immunotherapy (AIT) in comparison to healthy non-atopic controls and allergic patients tolerating AIT. Regulatory T cells (T regs), Th2 cells and innate lymphoid cells (ILCs) panels will be analyzed by flow cytometry.

2. To characterize metabolic and lipidomic profile of memory T regs, T effector cells, as well as different subpopulations of ILCs in the above-mentioned populations. Sorted cells will be processed to extract their lipidomic and metabolomic profiles by mass spectrometry.

CHANGES FROM THE PROPOSAL

This project changed the focus of the study from Macrophages and monocytes to T cells. The rationale behind is that the co-supervisor at the host institute found transcriptional changes in T cells in allergy patients in comparison to controls prior to the start of the fellowship (manuscript in preparation). Consequently, we decided to shift the focus of the study to T cell phenotyping instead of macrophages.

RESEARCH METHODOLOGY

Patients

Adult patients (18-55 years) that matched the following criteria were enrolled to the study before the start of the fellowship:

a) Residents in Cordoba for at least 10 years
b) They presented moderate or severe rhinoconjutivitis and/or asthma (following ARIA and GEMA criteria) during olive pollen season consecutively during 2 years.
c) Sensitized to olive allergen as demonstrated by an IgE titer > 15 kU/L to either major (Ole e1) and/or minor allergens (Ole7/Ole9).
d) Never received AIT treatment for olive or other allergies.
e) Agreed to participate in the study by signing an informed consent.

Exclusion criteria:

-Pregnant women (1 patient drop off the study as a consequence)

- Patients who do not have the capacity to understand their participation in the study

- Patients who do not agree to sign the informed consent

Patients were then divided in 3 different groups according to their sensitization to olive allergens: Controls (no sensitization, N=8), mild patients (patients with sensitization to Ole e1 >15kU/L with minor sensitization to Ole e7, N=4) and severe patients (patients with sensitization to Ole e1 and Ole e7 >15kU/L, n=13).

Four healthy individuals were also enrolled at Davos to optimize some protocols.

Sample collection

Outside of pollen season, we collected at least five tubes of fresh blood (4 tubes of 10 ml and 1 tube of 1 ml) from all the study subjects. The small tube was used to collect serum for a different purpose other than this fellowship. The rest was used to isolate the PMBCs used in the fellowship. PMBCs were then frozen and transported to Davos upon the start of the fellowship if they were collected in Spain.

Immunophenotyping and cell sorting

PMBCs were stained with an antibody panel develop to detect ILCs, T regs and Th2 cells. PMBCs from the different classes of patients and controls will be thawed and resuspended in RPMI medium. Then the cells were stained with the proper primary antibody for 20 minutes at 4°C and measured by flow cytometry and sorted depending on the phenotype (34-36) Table 1. The cells were sorted accordingly: T regs memory:(CD3+,CD4+,CD8-,CD45RA-,CD25+,CD127-); T effector memory:(CD3+,CD4+,CD8-,CD45RA-, CD25-,CD127+). Protocol optimizing samples were sorted to either PBS or RPMI medium. Samples from the patients were all sorted to RPMI medium. During the sorting process, samples were kept at 4ºC all time to ensure minimal disturbance of their metabolism.

Metabolomic conservation of samples

After sorted, samples were centrifuged at 370g 4ºC for 7 minutes. Supernatant was resuspended in 100 µl of methanol LC/MS grade (Fisher Scientific) and transferred to -80ºC until transfer to my home institute in Spain.

Marker	Dye	Isotype	Clone	Company
CD1a	FITC	M IgG1k	HI149	Biolegend
CD11c	FITC	M IgG1k	3.9	Biolegend
CD34	FITC	M IgG1k	581	Biolegend
CD94	FITC	M IgG1k	DX22	Biolegend
CD123	FITC	M IgG1k	6H6	Biolegend
CD303	FITC	M IgG2ak	201A	Biolegend
FceR1a	FITC	M IgG2bk	AER-37	Biolegend
CRTH2	PE	Rat, IgG2a,k	BM16	BD bioscience
CD117(c-kit)	PE-CF594	M IgG1k	YB5.B8	BD bioscience
CD8	PerCPCy5.5	M IgG2ak	SK1	Biolegend
Cd45RA	PE/Cy7	M IgG2bk	HI100	BD bioscience
CD161	AF647	M IgG1k	HP-3G10	Biolegend
CD4	AF700	M IgG1k	SK3	Biolegend
CD14, Viability dye AF780	APC-Cy7	M IgG1k	HCD14	Biolegend
CD16	APC-Cy7	M IgG1k	3G8	Biolegend
CD19	APC-Cy7	M IgG1k	HIB19	Biolegend
CD25	BV421	M IgG1k	BC96	Biolegend
CD3	V500	M IgG1k	UCHT1	BD bioscience
CD127	BV605	M IgG1k	A019D5	Biolegend
PD1	BV785	M IgG1k	MIH18	BD bioscience

Table 1. Antibody panel used to immunophenotype and sort the samples.

Sample treatment

Samples from four healthy individuals were used to optimize a protocol. Different numbers of CD3+ cells were sorted to test the limit of detection of the technique. Briefly, samples were weight and a mixture of methanol (MeOH)/ methyl tert-butyl ether (MTBE) 4:1. The volume depended on the weight of the pellet. Samples were then freeze/thaw by introducing them to 3 cycles of liquid nitrogen/ cold water bath for 10 seconds to break the cellular membrane. Then the samples were sonicated at 15W for 6 minutes to disrupt the nuclear and mitochondria membranes. Finally, samples were centrifuged at 16000 g at 4ºC for 10 minutes and the supernatant was collected to be measured by liquid chromatography coupled to mass spectrometry (LC-MS).

Quality Control (QC) sample preparation and randomization

QC samples were prepared by pooling equal volumes of sample from all samples. The QC sample was divided into different aliquots to preserve it from freezing-thawing cycles. QC samples followed the same procedure applied for the experimental samples. QC samples were analyzed throughout the run to provide a measurement of system stability, performance and the reproducibility of the sample treatment procedure. All samples were prepared randomly before metabolite extraction and for the corresponding analytical run.

Lipidomics

Supernatant of the processed samples were measured by a quadrupole LC-MS qTOF 6545 (Agilent). Briefly, 2 microliters of the sample were injected into a Poroshell 120 EC-C8 column (150 x 2.1 mm, 2.7 μm; Agilent), maintained at 60ºC. The flow rate was set at 0.5 ml/min. The data from electrospray ionization (ESI) were acquired in both positive (+) and negative (-) modes. The elution gradient involved a mobile phase consisting of: A) 0.1% ammonium formate (+) or 0.1% formic acid (-) in water, B) 0.1% ammonium formate (+) or 0.1% formic acid (-) in a mixture of methanol: isopropanol (85%:15%). The initial conditions set were 75% of phase B, which increase until 96% in 23 minutes. That concentration was maintained for 8 minutes. Then the rate increase in half a minute until 100% of phase B for cleaning the column. It was maintained for on minute before re-equilibration for 30 seconds. Finally, it was held at initial conditions for 7 minutes. The capillary voltage was set at 3500V in both modes. The drying gas flow rate was 11 L/min in + and 13 L/min in - at 290 °C. The gas nebulizer was set at 40 psi; fragmentor voltage was 175 V; skimmer and octopole radio frequency voltage (OCT RF Vpp) were set to 75 and 750 V. Data were collected in the centroid mode at a scan rate of one spectrum per second. Mass spectrometry window detection was performed in full scan from 50 to 1000 *m/z* for both modes. Reference masses for

ESI (+) were purine (*m/z* =121.0508) and HP-0921 (*m/z*= 922.0097), whereas for ESI (−) TFA NH4 (*m/z*= 119.0363) and HP-0921 (*m/z* =966.0007).

Data treatment

Acquired data were processed to provide structured raw data in an appropriate format for data analysis. Collected data from both batches were put together and cleaned of background noises and unrelated ions using Mass Hunter Profinder (B.08.00; SP3, Agilent Technologies) software. Molecular feature extraction (MFE) algorithm was used to reduce the size and complexity of data. Furthermore, after MFE, Find by Ion (FbI) algorithm was applied to improve the reliability in finding the features in the data, obtaining 592 chemical signals for LC-MS negative ionization mode and 540 for positive ionization mode. Raw data filtering was performed by removing those features detected in < 50 % of all QC samples.

RESULTS

Immunophenotyping and cell sorting

PMBCs from patients and controls were isolated from fresh blood in Cordoba (Spain) and Davos (Switzerland) and frozen. The samples from Spain were then sent to the host institute in Davos. An antibody panel was developed to detect different subsets of T cells (T regs, Th2 and T effector cells) as well as different ILC populations (ILC2 and ILC3) **Table 1.**

Then, PMBCs were thawed, stained with the antibody cocktail and analyzed by flow cytometry. At the same time, 2 populations were being sorted: T effector memory cells (Exclusion -, CD3+, CD4+, CD8-, CD45RA-, CD25- and CD127+) and T regulatory (T regs) memory cells (Exclusion -, CD3+, CD4+, CD8-, CD45RA-, CD127-, CD25+) **Figure1**.

To prevent changes in cell metabolism or prevent metabolite degradation sorted cells were centrifuged, resuspended and fixated in 100 μl methanol LC/MS grade. Cells then were also kept at -80ºC until transportation.

Metabolic profiling

For metabolomics, it is usual to use either biopsies, plasma or serum as those types of sample present a high quantity of metabolites that surpasses the limit of detection by a big margin. Because we had very limited amount of sorted cells (T effector cells around 750.000 cells per sample, T regs around 100.000) we needed to discern the limit of detection of our metabolomics protocol before measuring the patients' samples. To do that we sorted different numbers of T cells and our target populations from healthy individuals. Cells followed the same process described above. Upon receiving them at home institute metabolites were extracted using a mixture of methanol: MTBE (4:1) and by disrupting the different cellular membrane by snap freezing and sonication. Cell debris was then remove by centrifugation and the supernatant was measured by LC/MS.

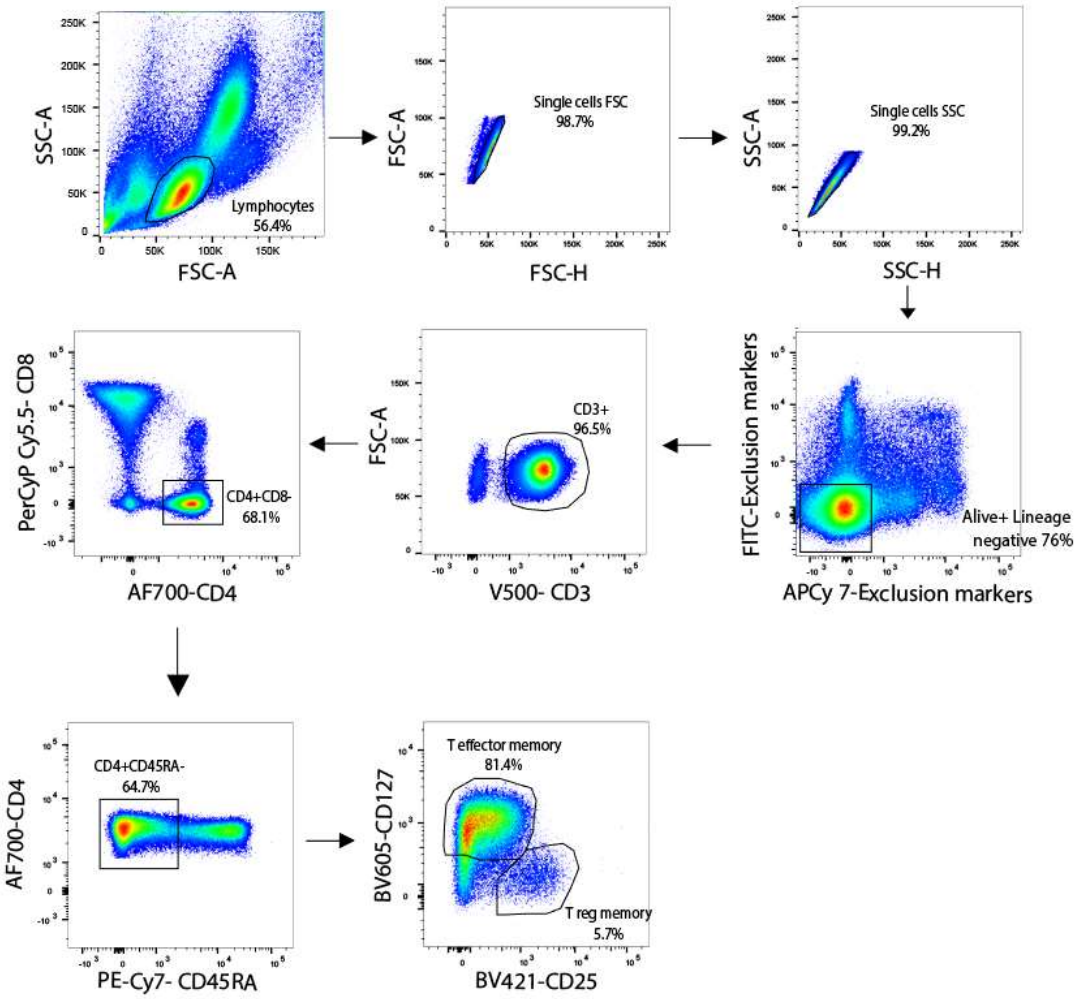

Figure 1. T effector memory cells and T regs cells sorted for metabolomic profiling. Representative figure of the gating strategy to sort T regs and T effector memory cells. First, lymphocytes were gated followed by two different gates to exclude doublets. Then cells that did not express any exclusion marker were selected and gated for CD3 to isolate T cells. Afterwards CD4+ and CD45- were selected. Finally, T regs memory were sorted as CD25+,CD127- and T effector memory cells as CD127+, CD25-. FITC exclusion markers: CD1a, CD11c, CD34, CD94, CD123, CD303 and FceR1a. APC-Cy 7 exclusion markers: Viability dye AF780 staining, CD14, CD16 and CD19.

The different numbers of T cells were measured in ESI+ mode and we obtained 540 chemical signatures. Representative metabolomic profiles are shown in the Figure 2. The best resolution was found in the sample of $750*10^6$ cells. Slightly lower, although with minor difference in between them, are the sample with 500 and $250*10^6$ cells. In the case of the sample with $100*10^6$ the resolution for most compounds is quite low, which makes them difficult to distinguish from the background. Therefore, we believe that the limit of detection of our technique is $250*10^6$ cells, meaning that we will have to combine patients to analyze the T regs compartment.

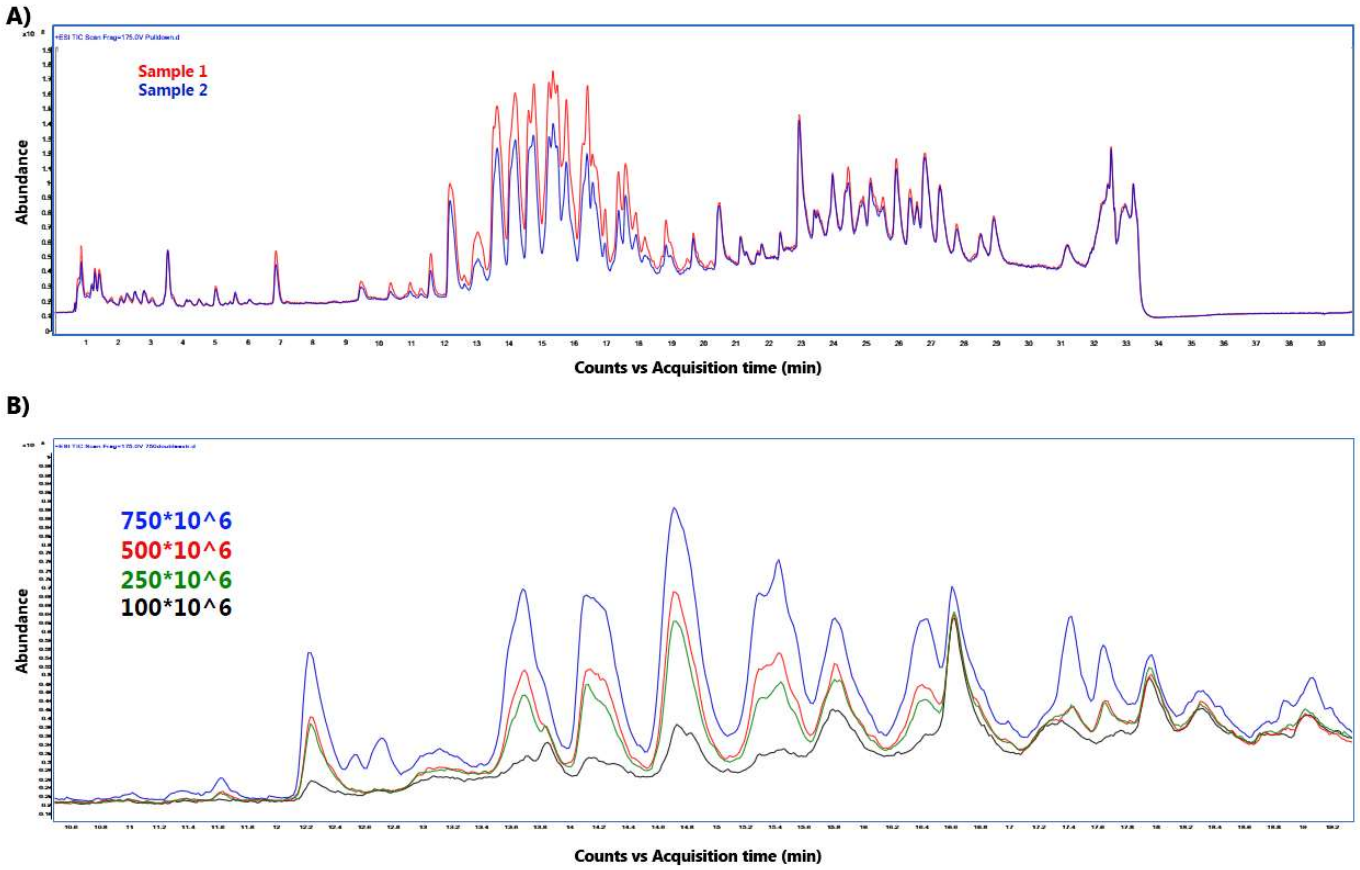

Figure 2. Lipidomic profile of control samples reveals the limit of detection of the technique in $250*10^6$ cells. A) Representative figure of the lipidomic profiles of two samples: One in red the other one in blue. B) Lipidomic profile of samples with different number of cells. Every peak belongs to a different chemical signal. In blue $750*10^6$, in red $500*10^6$, in green $250*10^6$ and in black $100*10^6$ cells.

Next, the target populations were measured in ESI-, as it is the technique in which lipid compounds are more difficult to detect. We obtained 592 chemical signals. A principal component analysis (PCA) was performed to detect differences between the two types of populations. There was no difference in chemical signals between populations in healthy

individuals (Figure 3A). However, distinctive signals were detected between both populations (Figure 3B).

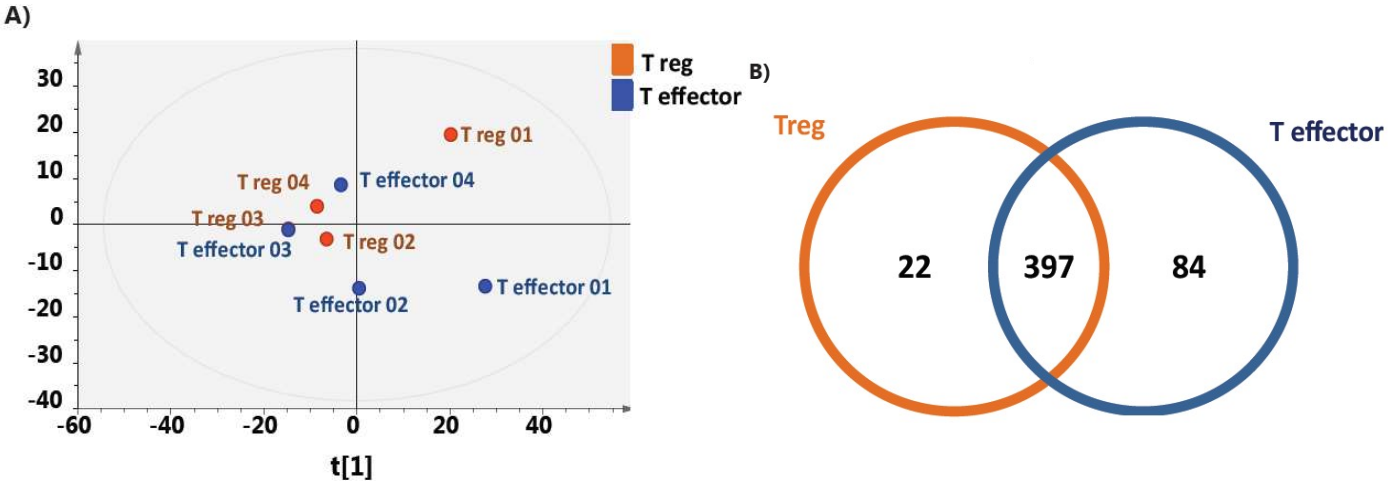

Figure 3. T regs and T effector cells present specific chemical signals in healthy individuals. A) Representative figure of the lipidomic profiles of two samples: One in red the other one in blue. B) Lipidomic profile of samples with different number of cells. Every peak belongs to a different chemical signal. In blue $750*10^6$, in red $500*10^6$, in green $250*10^6$ and in black $100*10^6$ cells.

FUTURE EXPERIMENTS

Analysis of the immunophenotyping is ongoing. Metabolic fingerprinting will be performed as planned at the home institute in May-August 2019 and the analysis will be finished before the end of the year. Depending on the results, functional assays with T effectors or T regs will be performed in the beginning of 2020 and shortly after the manuscript will be submitted.

CONCLUSIONS

This study focused on the metabolism of different T cell subsets and how it is disrupted in allergy. To do that we obtained PMBCs from very specific severe patients who usually do not respond to AIT and present a higher risk to develop food anaphylaxis (10-13). Then we immunophenotyped them focusing on the T cell and ILC compartment. Finally, we were able to analyze the metabolic profiling of sorted human T cells for the first time.

Further analyses are required to understand the impact of T cell metabolism in the allergic disease.

IMPACTS AND BENEFITS OF THIS STUDY

We have two major objectives with this study. The first one to study the metabolism of human T reg and T effector cells *ex vivo* in both healthy and allergic patients. The second one was to discover a new biomarker that could stratify patients who will not respond to AIT treatment. If successful, our study could help lessen the economic burden as well as improve patients' enrollment for AIT treatment. Furthermore, it could open the door to new intervention strategies to treat AIT unresponsive patients.

ADDITIONAL FELLOWSHIP ACTIVITIES

Courses:

i) Antibody panel design for flow cytometry by Dr. Sandra Blaszkiewicz (BD)

Seminars:

i) Moving from Bulk NGS to Precision Sequencing with Single-Cell Genomics: Resolving Heterogeneity in Blood and Solid Tumors by Dr. Engelbert Precht (Mission bio)

ii) 4th traditional Boyman Lab meets Akdis Lab

Congress participation:

i) WIRM XIII 2019 organizing committee member (IT group)

ii) WIRM XIII 2019 Workshop Seminar talk : Workshop session 4 "Immunometabolism of human T regulatory and T effector cells"

PERSONAL REFLECTION & ACKNOWLEDGEMENTS

I want first to thank EAACI for awarding me the mid-term Research Fellowship 2018, which allowed me to not only performed my project, but also to visit an exceptional institute with great scientists.

I want also to personally thank Cezmi Akdis for giving me the opportunity to stay in a fellowship in SIAF as well as his support and his great scientific comments and insight. I would also like to thank my co- supervisor Milena Sokolowska not only for her stimulating scientific discussions and guidance, but also for pushing me out of my comfort zone to make me a better scientist. Besides my supervisors I would like to thank also all the people in SIAF for their help and making me pass a wonderful time working there.

During this research fellowship, I had to adapt to a new environment and I had an opportunity to learn new techniques such as flow cytometry, NGS data analysis, FACS.

Finally, I would like to encourage young scientist or early stage career scientist with great interest in the field of immunology and allergy to apply for EAACI research or clinical fellowships.

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