EAACI Fellowship Awards
Final Report

Title of the fellowship **Amoxicillin-specific B cell characterisation in betalactam allergic patients**

Fellowship Type Long term research fellowship

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Research fellow Ruben Fernandez-Santamaria

Supervisor Prof. Cezmi A. Akdis

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

Acknowledge

First of all, I would like to express my gratitude to my host supervisor, Prof. Cezmi Akdis, who allowed me to join to the "SIAF family". I also want to thank Prof. Mübeccel Akdis for her kindly help, support and advice during the stay. I cannot forget to thank Dr. Willem Van de Veen, for his invaluable work, patience, support, continuous advice and technical tips during these 12 months. I appreciate quite a lot our long meetings, discussions and even some of the project-related headaches. I would also like to thank all SIAF members for their warm welcome, continuous support and social events, even during the hard times because of the COVID-19. I hope this stay is just the beginning of a longer collaboration between us. Finally, I would like to thank EAACI for giving me this wonderful opportunity, as it has been a crucial stage for my personal life and professional career.

What questions were addressed and why?

Betalactam (BL) antibiotics are the most frequent drugs used to treat bacterial infections, being amoxicillin (AX) the most commonly consumed antibiotic in Europe.[1] AX is also the most frequent BL involved in the induction of immediate hypersensitivity reactions (IDHRs).[2] in particular those mediated by immunoglobulin E (IgE) from those with a known immunological mechanism.[3,4]

Because of its low molecular weight, AX, as the rest of BL, needs to bind to proteins to be efficiently recognised by the immune system, according to the hapten hypothesis.[5-7] The multivalent compounds composed by several AX bound to the carrier protein are recognised by the immune system and able to trigger a hypersensitivity reaction. Human serum albumin (HSA), the most abundant protein in serum, has been the most important candidate as protein

carrier, although other proteins such as transferrin and heavy and light chains of immunoglobulins can also be important in the haptenisation process.[6,7]

During the development of IgE-mediated reactions, two different phases occur: a first step of sensitisation, with the production of drug-specific IgE and binding to mast cell on basophil surface , followed by a later phase of effector response, with the activation and degranulation of these cells.[8] Most studies have focused on the study of the effector phase, and little is actually known about the specific immunologic mechanisms involved during the first steps of the sensitisation phase,[9,10] as well as the differential B cells response between allergic patients and tolerant subjects. Although circulating drug/allergen-specific B cells are present at low rates, identifying the specific immunogenicity of this cell population is a crucial step to better understand the mechanism involved during the sensitisation phase which drives a tolerant or hypersensitivity response.[11,12]

Because of all this, the main objective of the study was the characterisation of AX-specific B cells from BL allergic patients and tolerant subjects. Concretely, we wanted to i) identify and characterise AX-specific memory and pre-plasma B cells, ii) Analyse their differential cytokine profile, assessing the differential immunoglobulin isotype production of AX-specific B cells, and iii) follow *in vivo* of AX-specific memory B cell clones.

Addressing these questions is of importance to better understand the role of B cells in the sensitisation phase during the development of an IDHR, as well as determining differences in terms of B cells development and immunoglobulin production between allergic patients and tolerant subjects.

What was the nature of the research?

This research was born from a close collaboration between the home institution, the Allergy Research group, from Instituto de Investigacion Biomedica de Malaga (IBIMA), Malaga, Spain, and the B cell Immunology group from the Swiss Institute of Allergy and Asthma Research (SIAF), Davos, Switzerland. This research was proposed in order to establish a new line of collaboration between our institutions in the fields of drug allergy and B cells immunology.

The home institution provided blood and serum samples from twenty patients with confirmed selective IDHRs to AX, diagnosed following the European Academy of Allergy and Clinical Immunology (EAACI) guidelines.[13-15] Samples from allergic patients were taken at different timepoints from the reaction: in an earlier stage (visit 1), close from the acute phase after a confirmed diagnostic, and at three different timepoints with intervals of 6 months of difference between them. Blood and serum samples were also taken from ten tolerant subjects. Serum AX-sIgE, -sIgG and tIgE were measured by ImmunoCAP®. In house radio allergo

sorbent test (RAST) was also used to measure serum AX-sIgE. Moreover, basophil activation test (BAT) with AX at three different concentrations was carried out using whole blood from both allergic patients and tolerant subjects.

In order to identify AX-specific B cells, AX was directly labelled with two different fluorochromes (PECy7 and Dy633). Due to the possibility of impairing the AX recognition by BCR receptors on B cells, because fluorescent dyes interact with the unique free amine group on the side chain of AX, different carriers: human serum albumin (HSA), poly-L-lysine (PLL), poly(amidoamine)-dendrimers (PAMAM) and polyethylene glycol (PEG)-spaced-bidendrons were firstly coupled to the same fluorescent dyes, and later on to AX. The recognition of these compounds was evaluated by using AX-specific hybridomas by flow cytometry and their recognition by AX-sIgE was done by RAST inhibition assays.

Serum AX-IgA1, -IgA2, -IgG1, -IgG2, -IgG3 and -IgG4 were measured by Enzyme-Linked ImmunoSorbent Assay (ELISA) from all visits of allergic patients and the tolerant subjects.

The frequency of different immunoglobulin subclasses producing-B cells was characterised by flow cytometry. AX-specific B cells were also identified using the fluorescent AX, and the different immunoglobulin isotype production from these specific AX-B cells was also assessed by flow cytometry. The frequency of B regulatory cells (Bregs), concretely, $CD19^{+}CD1d^{high}$, Br1, immature B cells, plasmablasts and B10 cells, with special interest in those which express Tim-1 and IL1RA and release IL-10, were analysed, including all visits of allergic patients and tolerant subjects by flow cytometry.

Switched B cells ($IgM^{-}IgD^{-}$) from allergic patients and tolerant subjects were sorted and immortalised by using a protocol well-established by the host group. After growing, single AX-specific B cell clones were selected using the fluorescent AX. Moreover, the immunoglobulin isotype production of each clone was assessed by flow cytometry. AX-IgG from the supernatant was measured by ELISA. Ribonucleic acid (RNA) was isolated from AX-specific and non-specific B cell clones.

What was the result?

Interesting results were obtained from the measure of the AX-sIgE, by both ImmunoCAP and in house RAST, in which higher serum AX-sIgE was observed in allergic patients compared to tolerant subjects. These results are in agreement with BAT results in which 56% of allergic patients but no healthy controls had positive results. A reduction of serum sIgE was detected 6 months after visit 1, and it continued reducing in the following visits, supporting the idea of sIgE clearance with time after the exposure to the drug. Interestingly, no difference was observed in the levels of serum AX-IgG between patients and tolerant subjects, indicating that both groups were exposed to the drug, although, unlike

tolerant subjects, allergic patients developed a hypersensitivity reaction. Although no difference was observed in AX-IgG levels, interesting preliminary data may have indicated a different AX Ig isotype production between allergic patients and tolerant subjects, as well as a different isotype immunoglobulin production in allergic patients based on the time passed from the acute phase.

Regarding the AX compound recognition, we obtained that, although in a different way, fluorescent PAMAM-AX, PLL-AX and AX were recognised by AX-specific hybridomas, whereas no recognition was observed with HSA-AX and PEG-AX. Preliminary data carried out in PBMCs from allergic patients and tolerant subjects indicated that PLL-AX were slightly unspecific, whereas PAMAM-AX seemed to interfere with the measure of other optical parameters in the flow cytometer. Because of this, it was decided to use fluorescent AX for the detection of specific B cells.

The preliminary results from B cell characterisation did not show big differences in the general population of B cells, based on its immunoglobulin isotype production between allergic patients and tolerant subjects. The inclusion of double-fluorescent AX allowed to detect specific AX-B cells, mostly in allergic patients. The specificity of the staining was assessed by blocking the BCR receptor on B cells, by adding free AX before culturing with the fluorescent-AX. Nevertheless, the fluorescent signal was extremely low in most cases, indicating a non-optimal recognition of fluorescent-AX by B cells. This low signal had made the later analysis of AX-specific B cell isotype production, as well as the correct isolation of these specific AX-B cells, needed to generate AX-specific B cell clones, very difficult. Finally, interesting differences were observed in the percentage of the different Breg cell populations between allergic patients and tolerant subjects, as well as between allergic patients at different timepoints. Nevertheless, an in-depth analysis of the data is being performed at the moment.

During the stay, two different manuscripts were published:

- Ogulur I, Pat Y, Ardicli O, Barletta E, Cevhertas L, Fernandez-Santamaria R, Huang M, Bel Imam M, Koch J, Ma S, Maurer DJ, Mitamura Y, Peng Y, Radzikowska U, Rinaldi AO, Rodriguez-Coira J, Satitsuksanoa P, Schneider SR, Wallimann A, Zhakparov D, Ziadlou R, Brüggen MC, van de Veen W, Sokolowska M, Baerenfaller K, Zhang L, Akdis M, Akdis CA. Advances and highlights in biomarkers of allergic diseases. Allergy. 2021 Sep 14. doi: 10.1111/all.15089. Epub ahead of print. PMID: 34519063.

- Fernandez-Santamaria R, Satitsuksanoa P. Engineered IL-10: A matter of affinity. Allergy. 2021 Oct 9. doi: 10.1111/all.15132. Epub ahead of print. PMID: 34626499.

How will the findings impact future research?

B cells are the main important cell population involved during the sensitisation phase of IDHRs, essential to better understand the specific mechanisms responsible for triggering a tolerant response or an IDHR. Although the role of B cells has been widely investigated in other allergies and during immunotherapy, no previous studies are available in the context of drug allergy. For this reason, this research and findings are of importance to improve our knowledge about these reactions and the immunological mechanisms and factors involved in their development.

The characterisation of the specific B cells, which can recognise AX and determine their concrete immunoglobulin production and BCR differences between allergic patients and tolerant subjects, would be essential to improve our knowledge about the specific role of B cells during the sensitisation phase of IgE-mediated IDHRs.

Personal reflection

This fellowship was an important step in my professional career, as basic researcher focused on the study of drug allergic diseases, with special interest in IgE-mediated hypersensitivity reactions to BL. The experience obtained during this stay has complemented my knowledge about allergic diseases and the immunological mechanisms involved. In addition, it gave me a broad and deep view of allergy, immunology, and science in general. Moreover, I had the opportunity to familiarise with novel and highly sophisticated laboratory techniques that have improved quite a lot my professional skills.

This stay gave me the chance to work during 12 months with top-scientists, enriching me both in a professional and personal way. The fluent interaction with SIAF members during the year, as well as the numerous discussions related to the project, significantly improved the research proposal and numerous new ideas have arisen, with important implications for future research and collaborations. Finally, I had the opportunity to share my work, meet other scientists, and learn first-hand from experts in the field thanks to attending to the 14th and 15th World Immune Regulation Meetings (WIRM).

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