

SUPPLEMENTARY MATERIAL

Methods

Skin tests

Intradermal tests were performed in the AP group as part of the inclusion criteria, using Pharmalgen (ALK Abelló SA, Madrid, Spain) at increasing concentrations from 0.00001 to 0.1 µg/mL. A positive result was established as the lowest concentration producing a wheal with mean diameter ≥ 5 mm [14].

Total serum IgE, sIgE and sIgG4 levels

Total serum IgE levels were measured by sandwich immunoassay on an Advia Centaur analyser (Siemens Healthcare, USA); sIgE and sIgG4 levels to AmV, rApi m 1 (phospholipase A2), rApi m 2 (hyaluronidase), rApi m 3 (acidic phosphatase), rApi m 5 (dipeptidyl peptidase IV) and rApi m 10 (icarapin) were measured by fluoroimmunoassay with ImmunoCAP 250 (Thermofisher, Uppsala, Sweden), according to the manufacturer instructions. In order to quantify the sIgE and sIgG4 levels to Api m 4, melittin sequence H-GIGAVLKVLTGTPALISWIKRKRQQ-OH (Schafer-N ApS, Denmark) was coupled to activated CAPs by Thermofisher Scientific [15].

Tryptase

Baseline serum tryptase levels of all patients were measured by fluoroimmunoassay with ImmunoCAP 250 (ThermoFisher, Uppsala, Sweden). This value was necessary to calculate the REMA Score to assess the risk of mastocytosis [16].

Basophil activation test

The basophil activation test (BAT) using heparinized whole blood samples was performed with AmV (Pharmalgen, ALK-Abello, Madrid, Spain) at 0.1 µg/mL and 1 µg/mL, as previously described [17]. Phosphate buffered saline (PBS) was used as a negative control and N-Formyl-Met-Leu-Phe (f-MLP, ref. f3506 Sigma-Aldrich) as a positive control. Cell staining was performed using CD63-FITC/CD123-PE/anti-HLA-DR-PerCP antibody cocktail (ref. 341068, BD FastImmune™, Becton, Dickinson and Company, San Jose, CA, USA). Acquisition was performed in a BD FACS Canto II cytometer (Becton Dickinson and Company, San Jose, CA, USA), using BD FACS Diva™ as acquisition and analysis software. At least 500 CD123⁺ events were recorded. Basophil degranulation was measured as the percentage of basophils expressing CD63 (%CD63⁺) and considered as a continuous variable (Supplementary Figure 1.A).

Identification of Th1/Th2/Th17 cell subpopulations

Identification of CD4⁺ T-cell subpopulations was performed by intracytoplasmic staining according to their cytokine secretion pattern, using the Human Th1/Th2/Th17 Phenotyping Kit (ref. 560751, BD Pharmingen™, Becton, Dickinson and Company, San Jose, CA, USA). To that end, 1:1 PBS-diluted heparinized whole blood samples were

stimulated for 5 hours at 37°C and 5% CO₂ using phorbol 12-myristate 13-acetate (ref. P1585, Sigma-Aldrich) at 50 ng/mL and ionomycin calcium salt (ref. I0634, Sigma-Aldrich) at 1 µg/mL in the presence of BD GolgiStop™ Protein Transport Inhibitor (provided in the kit). AmV (Pharmalgen, ALK-Abello, Madrid, Spain) at 0.1 µg/mL or 1 µg/mL was added to the cell culture.

After the stimulation, cells were collected, fixed and permeabilized before staining, according to the manufacturer instructions. Staining was performed using the CD4-PerCP-Cy5.5/IL-17A-PE/INF-GMA-FITC/IL-4-APC cocktail included in the kit (Supplementary Figure 1. B). Sample acquisition was performed in a BD FACS Canto II cytometer (Becton Dickinson and Company, San Jose, CA, USA), using BD FACS Diva™ as acquisition and analysis software. At least 20,000 CD4⁺ lymphocytes were acquired.

Identification of regulatory T cell subpopulations

Peripheral Treg cells were phenotyped from heparinized whole blood samples. To that end, 100 µL of whole blood was mixed with 50 µL of Brilliant Stain Buffer (ref. 566349, BD Horizon, Becton, Dickinson and Company, San Jose, CA, USA) and stained using the Transcription Factor Buffer Set kit (ref. 562574, BD Pharmingen™, Becton, Dickinson and Company, San Jose, CA, USA) according to the manufacturer protocol. The following combinations of monoclonal antibodies were used:

- Tube 1: CD3-BB515 (ref. 564465, BD Horizon), CD4-APC-H7 (ref. 560158, BD Pharmingen), CD25-PE-Cy7 (ref. 557741, BD Pharmingen), CD127-Alexa647 (ref. 558598, BD Pharmingen), CD39-BV421 (ref. 563679, BD Horizon), CD45RA-BV510 (ref.

563031, BD Horizon), CTLA-4-PE (ref. 555853, BD Pharmingen) and CD8-PerCP-Cy5.5 (ref. 565310, BD Pharmingen).

- Tube 2: CD3-BB515 (ref. 564465, BD Horizon), CD4-APC-H7 (ref. 560158, BD Pharmingen), CD25-PE-Cy7 (ref. 557741, BD Pharmingen), CD127-Alexa647 (ref. 558598, BD Pharmingen), Ki-67-BV421 (ref. 562899, BD Horizon) and Helios-PE (ref. 563801, BD Pharmingen).

Acquisition was performed in a BD FACS Canto II cytometer (Becton Dickinson and Company, San Jose, CA, USA), using BD FACS Diva™ as acquisition and analysis software. At least 20,000 CD4⁺ lymphocytes were acquired (Supplementary Figure 1.C).

Cytokine IL-4 and IL-10 production

The production of IL-4 and IL-10 were measured in the supernatant of the cell culture performed with 1:1 PBS-diluted heparinized whole blood samples stimulated for 24 hours at 37°C and 5% CO₂ using phorbol 12-myristate 13-acetate (ref. P1585, Sigma-Aldrich) at 50 ng/mL and ionomycin calcium salt (ref. I0634, Sigma-Aldrich) at 1 µg/mL. AmV (Pharmalgen, ALK-Abello, Madrid, Spain) at 1 µg/mL was added to the cell culture. After centrifuging, the supernatant was collected and frozen at -80 °C for further processing.

Released IL-4 and IL-10 were quantitated using the customized Milliplex® Map Human High Sensitivity T Cell Magnetic Bead Panel (ref. HSTCMAG-28SK, Millipore Corporation, USA), following the manufacturer instructions. Samples were acquired in a Luminex platform (LABScan 100) using xPONENT v4.2 as acquisition and analysis software.

Statistics

Demographic characteristics of patients were expressed as mean \pm standard deviation (SD) or median (maximum, minimum) for continuous variables, and as frequency distribution and proportions for categorical variables. All the cellular variables were expressed as median (maximum, minimum). The Kruskal-Wallis test was used to determine the overall differences between the three groups. The Student's t-test analysed the differences between the groups two by two for parametric variables and the Mann-Whitney test for nonparametric variables. To see the correlation between quantitative variables, Pearson's correlation coefficient was used. Post-hoc tests were also performed for multiple comparisons using Bonferroni Holm correction of p-values [18]. Heatmaps were used to visualize individual expressions and classify cellular variables. The *p*-values <0.05 were regarded as statistically significant. R statistical software (vs. 3.5.0) was used to perform all the analyses.