

## **Mast Cell Activation Profile and T<sub>FH</sub>13 Detection Discriminate Food Anaphylaxis Versus Sensitization**

**Short title:** Mast cell activation profile and T<sub>FH</sub>13 in food anaphylaxis

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## **ABSTRACT**

### **BACKGROUND**

The prevalence of food allergy (FA) has increased significantly, and the risk of developing anaphylaxis is unpredictable. Thus, discriminating between sensitized patients and those at risk of having a severe reaction is of utmost interest.

### **OBJECTIVE**

To explore mast cell activation pattern and T follicular helper (T<sub>FH</sub>) 13 presence in sensitized and food anaphylaxis patients.

### **METHODS**

Patients sensitized to Lipid transfer protein (LTP) were classified as anaphylaxis or sensitized depending on the symptoms elicited by LTP-containing food. CD34<sup>+</sup>-derived MCs from patients and controls were obtained, sensitized with pooled sera, and challenged with Pru p 3 (peach LTP). Degranulation, PGD<sub>2</sub>, and cytokine/chemokine release were measured. The T<sub>FH</sub>13 population was examined by flow cytometry in the peripheral blood of all groups. In parallel, LAD2 cells were activated similarly to patients' MCs.

### **RESULTS**

A distinguishable pattern of mast cell activation was found in anaphylaxis compared to sensitized patients. Robust degranulation, PGD<sub>2</sub>, and IL-8 and GM-CSF secretion were higher in anaphylaxis, whereas TFG- $\beta$  and CCL2 secretion increased in sensitized patients. Concomitantly, anaphylaxis patients had a larger T<sub>FH</sub>13 population. MC activation profile was dependent on the sera rather than the MC source. In agreement with that, LAD2 cells reproduce the same pattern as MCs from anaphylactic and sensitized patients.

### **CONCLUSION**

The distinct profile of mast cell activation allows to discriminate between anaphylaxis and sensitized patients. Pooled sera may determine mast cell activation independently of mast cell origin. Besides, the presence of T<sub>FH</sub>13 cells in anaphylaxis patients points to an essential role of IgE affinity.

**Key words:** Anaphylaxis. Food allergy. IgE. Inflammation. Mast cells. T<sub>FH</sub>13.

## RESUMEN

### BACKGROUND

La prevalencia de alergia alimentaria (AA) se ha incrementado de forma significativa, y el riesgo de desarrollar una anafilaxia es impredecible. Por este motivo, el poder discriminar entre individuos sensibilizados y aquellos en riesgo de desarrollar una reacción grave es de gran interés.

### OBJETIVO

Comparar el patrón de activación mastocitaria y la presencia de linfocitos T foliculares helper (TFH) 13 en individuos sensibilizados y con alergia alimentaria.

### METODOLOGIA

Pacientes sensibilizados a la Proteína de Transferencia de Lípidos (LTP) se clasificaron en dos grupos, anafilaxia y sensibilizados, en función de los síntomas inducidos por el consumo de alimentos con LTP. Se obtuvieron mastocitos derivados a partir de progenitores CD34+ tanto de pacientes como de controles, se sensibilizaron con el suero de los diferentes individuos, y se estimularon con Pru p 3 (LTP de melocotón). Se evaluó la desgranulación, los niveles de PGD<sub>2</sub>, y la liberación de citocinas/quimioquinas. La población TFH13 se examinó en sangre periférica mediante citometría de flujo en todos los grupos. En paralelo, células LAD2 se activaron del mismo modo que los mastocitos de los pacientes.

### RESULTADO

Se observó un patrón de activación mastocitaria diferencial entre los pacientes con anafilaxia y los sensibilizados. La desgranulación, la producción de PGD<sub>2</sub>, IL-8 y GM-CSF fue superior en el grupo de anafilaxia, mientras que la producción de TFG-b y CCL2 era superior en pacientes sensibilizados. De forma concomitante, los pacientes con anafilaxia presentaron una población mayor de TFH13. El perfil de activación mastocitaria fue dependiente del suero del paciente, más que del origen del mastocito. Igualmente, este patrón se pudo observar en el modelo de LAD2, tanto el observado en mastocitos de pacientes con anafilaxia como en los sensibilizados.

### CONCLUSION

Los pacientes con anafilaxia se pueden diferenciar de los sensibilizados gracias a la presencia de un patrón de activación mastocitaria distinto. Además, la mayor presencia de linfocitos TFH13 en los pacientes con anafilaxia sugiere un papel destacable de la afinidad de la IgE en el desarrollo de la anafilaxia.

**Palabras clave:** Anafilaxia. Alergia alimentaria. IgE. Inflamación. Mastocitos. TFH13.

## SUMMARY BOX

Food allergy prevalence has increased significantly in the last decades, and currently, there is no available biomarkers to identify which patients with food sensitization are going to develop an anaphylaxis.

Mast cell activation profile and detection of T<sub>FH</sub>13 allows discrimination of patients at risk of anaphylaxis from those sensitized, helping in risk stratification in a complex model of food allergy such as LTP.

## INTRODUCTION

Food anaphylaxis has increased critically in the last decades [1–3]; however, the main prophylactic strategy is still limited to food avoidance. Food allergy (FA) diagnosis is frequently challenging due to the difficulties differentiating between sensitization and true allergy [4–6]. This is particularly problematic in patients sensitized to pan allergens often related to anaphylaxis, such as lipid transfer proteins (LTP) [7,8]. Thus, oral challenge tests are still considered the gold standard, although tools for accurate risk assessment are limited. Consequently, in some cases, patients may follow unnecessarily restricted diets that significantly impact their quality of life [9].

In recent years, the basophil activation test (BAT) in FA was shown to help distinguish clinically relevant from irrelevant sensitization [10–12]. In parallel, since mast cells (MCs) are considered the primary effector cells of allergy [13], an MC activation test (MAT) was developed and has already improved the diagnosis of IgE-mediated peanut allergy [14,15]. MCs are commonly obtained from peripheral blood by progenitor isolation and differentiation *in vitro* [15–19]. Other approaches involve the use of MCs derived from induced pluripotent stem cells [20], and, in a less laborious and time-consuming procedure, the human MC line LAD2 has also rendered good results [21].

Our understanding of the immune basis of FA has increased in recent years. Studies of food-allergic patients and murine models point to the affinity of IgE as a key factor related to MC degranulation and anaphylaxis [22–25]. T follicular helper (T<sub>FH</sub>) cells direct the affinity and isotype of antibodies synthesized by B cells; the nature of signals that switch to low versus high affinity may differ [26,27]. T<sub>FH</sub> cell-derived interleukin-4 (IL-4) is necessary for IgE production; however, IL-13-producing T<sub>FH</sub> cells are induced by allergens [28,29]. These T<sub>FH</sub>13 cells have a different cytokine profile (IL-13<sup>hi</sup>IL-4<sup>hi</sup>IL-5<sup>hi</sup>IL-21<sup>lo</sup>) and are required to produce high- but not low-affinity IgE. These cells are necessary for anaphylactic reactions; therefore, T<sub>FH</sub>13 cells may identify severe patients, as shown [30].

Along this line, MC mediators' release may depend on the affinity of IgE. The high-affinity IgE induces a stronger degranulation and a higher cytokine secretion than the low-affinity IgE, resulting in more elevated chemokine secretion in the latter [23,31]. Thus, differential cytokines and chemokines secretion may be involved in FA [32].

This study aims to identify factors that may differentiate patients at risk of anaphylaxis from those only sensitized. In two opposite phenotypes of LTP-sensitized individuals, i.e., patients with food anaphylaxis and sensitized individuals (with no symptoms), we compared (1) MC activation induced by peach LTP (Pru p 3) and (2) the presence of T<sub>FH</sub>13 cells in peripheral blood. Our results show that serum samples from anaphylaxis patients induce distinguishable MC activation patterns. The T<sub>FH</sub>13 cell population is more abundant in anaphylaxis patients than sensitized individuals, suggesting their potential use as a risk biomarker of severe reactions.

## MATERIALS AND METHODS

### Study population

Patients were recruited at the Allergy Department of the Hospital Clínic of Barcelona. Informed consent was obtained from all participating subjects. The study was approved by the local ethics committee of the Hospital Clínic (Barcelona, Spain).

Patients sensitized to peach LTP-Pru p 3 with specific IgE (sIgE) levels [3] 0.10 KU<sub>A</sub>/L (ImmunoCAP®, Thermo Fisher Scientific, Uppsala, Sweden), with no other sensitization identified were recruited. They were classified into two groups depending on the reaction severity upon peach ingestion: (1) *anaphylaxis* patients with a convincing history of anaphylaxis and (2) *sensitized* patients with no symptoms. The oral challenge was not performed in anaphylaxis patients[33], and a recent history of tolerance to peach was required in the sensitized group. Healthy volunteers with no respiratory or food allergies were also recruited as controls.

To carry out this study, the patients and healthy volunteers recruited were divided into two cohorts to develop the different in vitro studies: (1) a cohort to generate pooled sera for mast cell activation, PGD2 secretion and cytokine analysis assays and (2) a cohort to generate MCs and to detect T<sub>FH</sub>13.

Total IgE and Pru p 3-specific IgG4 (sIgG4) were measured by the ImmunoCAP® System (Thermo Fisher Scientific, Uppsala, Sweden).

### **CD34<sup>+</sup>- derived mast cell generation**

A total of 15 individuals were recruited: six anaphylactic, five sensitized, and four healthy volunteers (demographic and clinical characteristics are presented in Table 2). MCs from these groups were obtained from 100 ml of peripheral blood. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque (Sigma, St. Louis, MO, USA) following the protocol previously described[15,16]. Briefly, blood was diluted with PBS (Lonza Bioscience, Morrisville, USA), layered over Histopaque, and centrifuged at room temperature at 400 xg for 20 minutes. PBMCs were collected, washed, and incubated with MACS Buffer (0.5% BSA; 2 mM EDTA; 50 ml PBS) and the CD117 Microbead Kit Human (Miltenyi Biotec, Bergisch Gladbach, Germany). CD117<sup>+</sup> (or KIT) cells were selected using a magnetic field with LS columns (Miltenyi Biotec) and suspended with MC culture medium (StemPro-34 Medium; Thermo Fisher, Massachusetts, United States) supplemented with 1% penicillin-streptomycin (Lonza Bioscience), 1% L-glutamine (Lonza Bioscience), rh IL-6 (50 ng/ml; Immunotools, Friesoythe, Germany), and SCF (100 ng/ml; Immunotools). Rh IL-3 (10 ng/ml; Immunotools) was added at day 0 of the culture. MC culture medium was added to the cell culture every two weeks. At week 7, MCs were characterized.

### **Characterization of CD34<sup>+</sup>-derived mast cells**

To check the morphology,  $5 \times 10^4$  cells were centrifuged with Cytospin at 500 rpm for 5 minutes and then stained with May-Grünwald Giemsa.

For MC differentiation analysis,  $5 \times 10^4$  cells were taken from culture, blocked, and stained with APC-conjugated anti-Fc $\epsilon$ RI (BioLegend, San Diego, CA, USA) and PE-conjugated anti-CD117 (Santa Cruz Biotechnology, Dallas, Texas, USA). Cells were acquired on a FACSCalibur flow cytometer (FACScan; BD Biosciences, Mountain View, CA, USA) and analyzed using FlowJo software version 10.8.

The  $\beta$ -hexosaminidase assay was performed to check the functionality of CD34<sup>+</sup>-derived MCs. A total of  $6 \times 10^4$  cells were taken from culture and sensitized overnight with 0.1  $\mu$ g/ml biotinylated human IgE (Abbotec, San Diego, CA, USA) in triplicates into 96-well plates. Cells were stimulated with 0.4  $\mu$ g/ml Streptavidin (STV) (Sigma, St. Louis, MO, USA) for 30 minutes at 37 °C. Plates were subsequently centrifuged, and  $\beta$ -hexosaminidase was assayed in the supernatants and cell pellets as described[34,35]. Degranulation was calculated as the percentage of  $\beta$ -hexosaminidase recovered from the supernatants compared with total cellular content.

### **Mast cell activation and PGD<sub>2</sub> secretion**

We obtained serum samples from eight anaphylaxis and four sensitized patients. As early described, these sera were pooled based on similar ratios of Pru p 3 sIgE/total IgE levels (tIgE:sIgE). The characteristics of the individual donors and pooled sera are shown in Table 1.

Next,  $5 \times 10^4$  MCs or LAD2 cells, the latter kindly provided by Dr. D. Metcalfe (NIH, Bethesda, MD, USA) [36], were incubated with 10 ng/ml rh IL-4 (Immunotools) for 5 days [37,38] and sensitized overnight with pooled sera from the different groups (Table 1) diluted to obtain a total concentration of Pru p 3 sIgE = 1 KU<sub>A</sub>/L. Cells were washed and stimulated with 1 µg/ml Pru p 3 (Roxall, Trofa, Portugal) for 30 minutes at 37 °C. The supernatants were kept at -80 °C for later Prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) analysis using an ELISA kit (Cayman Chemical, Ann Arbor, Mich) as described [39]. Cells were blocked and stained with PE-conjugated anti-CD63 (BD Biosciences, Mountain View, CA, USA). Cells were acquired on a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA, USA) and analyzed using FlowJo software version 10.8. Experiments were performed in duplicate in patients where possible (limitation: low number of cells obtained).

### **Detection of T<sub>FH</sub>13 cells**

T<sub>FH</sub>13 cells were detected following the protocol previously described [29]. Briefly, PBMCs from patients and healthy volunteers were thawed, and CD4<sup>+</sup> T cells were isolated using the EasySep Human CD4<sup>+</sup>T Cell Enrichment Kit (Stemcell Technologies). CD4<sup>+</sup>T cells were incubated overnight with IMDM (Gibco) complete media (supplemented with 10% v/v heat-inactivated FBS, 100 U/mL penicillin-streptomycin, 2 mmol/L L-glutamine, 10 mmol/L HEPES, and 1 mmol/L sodium pyruvate). Then,  $1 \times 10^6$  cells were incubated with IMDM complete media, 50 ng/ml PMA (Sigma), and 1 µg/ml Ionomycin (Sigma) for 6 hours (after the first hour, Brefeldin A was added at 1:1000). After 6 hours, cells were stained with surface antibodies: PerCP-conjugated anti-CD3 (Immunotools), APC-conjugated anti-CD4 (Immunotools), PE-conjugated anti-CD45RA (Immunotools) and APC/Cyanine7-conjugated anti-CXCR5 (BioLegend), fixed with Fixation/Permeabilization Buffer (BD Biosciences, Mountain View, CA, USA) and incubated with Perm/Wash Buffer (BD Biosciences) overnight at 4 °C. Cells were then stained with intracellular antibodies: FITC-conjugated anti-IL4 (BioLegend), Brilliant Violet 421-conjugated anti-IL13 (BioLegend), and PE/Cyanine7-conjugated anti-IFNγ

(BioLegend). Cells were acquired on an Attune flow cytometer (Thermo Fisher Scientific, Uppsala, Sweden) and analyzed using FlowJo software version 10.8.

### **Western blotting**

After incubating with pooled sera from the different groups, we analyzed the intracellular activation pattern and activation with Pru p 3 in the LAD2 model. The adaptor protein LAT is critical in MC Fc $\epsilon$ RI signaling, linking the IgE high-affinity receptor to calcium influx and degranulation [40]. Western blotting was carried out as described [41,42]. LAD2 cells were sensitized with pooled sera 1:1 overnight and stimulated with 2  $\mu$ g/ml of Pru p 3. The reaction was stopped at 0, 30 seconds, and 2 minutes, and then cells were lysed. Total protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc. USA) according to the manufacturer's recommendations. Electrophoresis and protein blotting was performed using NuPage™ 4-12% Bis-Tris Gel, 1.5 mm\*15 w (Invitrogen, Waltham, Massachusetts, USA), and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Blots were probed with anti-pTyr:HRP (BD Transduction Laboratories, Mountain View, CA, USA), rabbit anti-pLAT (Cell Signaling Technology, Danvers, Massachusetts, USA), rabbit anti-LAT (Cell Signaling Technology) and goat anti-rabbit-HRP (Life Technologies, Carlsbad, CA). In all blots, proteins were visualized by enhanced chemiluminescence (WesternBright™ ECL, Advansta, USA).

### **Cytokine Multiplex Assay**

CD34<sup>+</sup>-derived MCs from peripheral blood from patients, healthy controls, and LAD2 were used to determine the mediator's release. MCs from patients (anaphylaxis and sensitized) and LAD2 were incubated overnight with pooled sera (anaphylaxis and sensitized, respectively). The next day, 1x10<sup>5</sup> cells were cultured in a 48-well plate and treated with 1  $\mu$ g/ml Pru p 3 (Roxall) for 24 hours at 37°C. Likewise, LAD2 cells were incubated with 0.1  $\mu$ g/ml biotinylated human IgE (Abbotec) and stimulated with 0.4  $\mu$ g/ml Streptavidin (STV) (Sigma). The supernatants were kept at -80°C for later cytokine and chemokine measurement using the ProcartaPlex Multiplex Assay (Invitrogen) as described before [43]. In the Multiplex Assay, 50  $\mu$ l of supernatant was combined with a panel of beads covalently bound to an antibody that recognized one of the following cytokines/chemokines: IL1- $\beta$ , IL-6, IL-8, IL-13, GM-CSF, TGF- $\beta$ , TNF- $\alpha$  and CCL2.

### **Statistical analysis**



Statistical analysis was performed using PRISM 9 (GraphPad Software, La Jolla, CA, USA). All results are expressed as mean  $\pm$  standard deviation (SD). One-way ANOVA was used to determine significant differences ( $p$ -value) between several experimental groups, and the T-test was used to determine significant differences ( $p$ -value) between two groups after determining the normal distribution of the samples and variance analysis.

## RESULTS

### Patient characteristics

The characteristics of the patients recruited to obtain (1) serum to create pooled sera (2) CD34<sup>+</sup>-derived MCs and T<sub>FH</sub>13 are listed in Tables 1 and 2, respectively.

Specific IgE (sIgE) values were higher in anaphylaxis than in sensitized patients, but the total IgE/specific-IgE ratio (tIgE/sIgE) was higher in sensitized patients (Table 1). The specific IgG<sub>4</sub> and IgE ratio (sIgG<sub>4</sub>/sIgE) was also higher in sensitized patients (Table 1). The tryptase level of the patients was measured to rule out tryptasemia (Table 2).

### Sera from anaphylaxis patients induce stronger degranulation and PGD<sub>2</sub> production in CD34<sup>+</sup>-derived MCs.

MCs from patients and healthy volunteers were differentiated (CD117<sup>+</sup>/Fc $\epsilon$ R1<sup>+</sup>) *in vitro* after seven weeks (Table S1), and their ability to degranulate to positive stimuli (PMA + Ionomycin and biotinylated IgE plus Streptavidin) was confirmed (Figure S1).

MCs from healthy controls, sensitized, and anaphylaxis patients were incubated overnight with pooled sera from healthy controls, anaphylaxis, or sensitized patients. The serum volume was corrected by the sIgE values. Afterward, cells were incubated with Pru p 3 for 30 minutes, and the surface expression of CD63<sup>+</sup> was measured using flow cytometry.

MCs from all three groups, healthy controls, anaphylaxis, and sensitized patients, had a significantly higher activation when sensitized with pooled anaphylaxis sera, compared with pooled sera from healthy volunteers or sensitized patients (Figures 1A, B, & C, respectively). Sera from sensitized patients induced similar degranulation to healthy individuals. Cells sensitized with sera with no Pru p 3 challenging did not activate (Table S2).

Next, we aimed to confirm our results by analyzing PGD<sub>2</sub> release under the same conditions. Again, MCs from all three groups, healthy controls, anaphylaxis, and sensitized patients, had a higher PGD<sub>2</sub> production when incubated with pooled anaphylaxis sera compared with pooled healthy volunteers or sensitized sera (Figures 2A, B, & C, respectively). However, it was only significant for MCs from sensitized and anaphylaxis patients, not from healthy individuals. Finally, a significant correlation between degranulation (CD63<sup>+</sup>) and PGD<sub>2</sub> synthesis was observed in all groups (Figure S2).

#### **Sera from anaphylaxis patients induce more robust activation in LAD2 cells.**

We performed the same experiments using LAD2 cells to correct the potential effect of MC phenotype on activation and to reproduce our observations in a different MC model. LAD2 cells sensitized with pooled sera from the anaphylaxis patients showed significantly higher degranulation than those incubated with sera from healthy and sensitized individuals (Figure 3A). As shown in Figure 3B, a pattern of phosphotyrosine proteins was induced, with increased LAT phosphorylation in anaphylaxis patients compared with the sensitized patients, in agreement with the higher degranulation (Figure 3A).

#### **T<sub>FH</sub>13 cells are more abundant in anaphylaxis patients.**

Our results show the marked ability of sIgE in the anaphylaxis pool to yield a greater mast cell degranulation. Recently, T<sub>FH</sub>13 cells have been found to regulate the induction of high-affinity IgE [30]. Thus, we investigated the presence of the T<sub>FH</sub>13 population in PBMCs following the gating strategy shown in Figure S3 and previously described [29].

We observed that patients from the anaphylaxis group showed a significantly higher number of T<sub>FH</sub>13 cells than the other groups. Sensitized patients presented similar results to healthy individuals. Indeed, we identified a significant correlation between degranulation (CD63<sup>+</sup>) and T<sub>FH</sub>13 presence (Figure 4).

#### **Sera from anaphylaxis patients induce a higher pro-inflammatory pattern in CD34<sup>+</sup>-derived MCs and LAD2 cells.**

Thus, in this study, we found that MCs from the anaphylaxis group incubated with pooled anaphylaxis sera produced more significant amounts of IL8 and GM-CSF (Figure 5A), having a higher pro-inflammatory profile than MCs from the sensitized group incubated

with pooled sensitized sera. Interestingly, the MCs from the sensitized group incubated with pooled sensitized sera produced more TGF- $\beta$  and CCL2 (Figure 5A), inducing a more protective profile. While we did not find significant differences between groups for the other cytokines (IL-1 $\beta$ , IL-6, IL-13, and TNF- $\alpha$ ) studied, there was a trend. MCs with pooled anaphylaxis sera produce more (IL-1 $\beta$ , IL-6, IL-13, and TNF- $\alpha$ ) than those incubated with pooled sera from sensitized patients (Figure S4).

Similarly, when LAD2 cells were incubated with pooled sera from the anaphylaxis patients or with biotinylated human IgE and activated with Pru p 3 or Streptavidin respectively, they produced more IL8 and GM-CSF. Conversely, when LAD2 cells were incubated with pooled sera from the sensitized patients and activated with Pru p 3, they had more TGF- $\beta$  and CCL2 (Figure 5B).

## DISCUSSION

Our study found that the mast activation profile could differentiate patients with severe reactions from those only sensitized to LTP. Our results are consistent with other studies involving mast cell activation test (MAT) and peanut as the allergen [14,15,21,44]. MAT is an *in vitro* diagnostic tool that combines the allergen, allergen-specific IgE, and human MCs—the three crucial elements of the effector phase of IgE-mediated allergic responses [45]. Interestingly, in our model, we found that the humoral component is far more critical than the cellular one in inducing MC degranulation and PGD<sub>2</sub> production, given that when LAD2 cells were used instead of CD34<sup>+</sup>-derived MCs from patients, the activation/degranulation patterns were unaltered. However, LAD2 showed lower degranulation than CD34<sup>+</sup>-derived-MCs from patients under the same conditions, as reported in other studies [46].

We found that sIgE levels were higher in anaphylaxis than in sensitized patients, which could account for the higher activation of the first group, as reported in other studies [21]. Issan *et al.*, [47] reported that MCs from healthy donors responded to high specific IgE levels but not to low ones. However, we normalized sIgE values for each group, sensitizing all MCs with the same amount of sIgE to eliminate this potential confounding factor. Therefore, our results suggest that MC responses may depend on other humoral factors, such as the affinity of IgE for the allergen, which could vary between individuals, as proposed in other studies [14].

Along this line, we have shown that T<sub>FH</sub>13 cells were more abundant in patients with anaphylaxis than those sensitized, indicating that they might have specific IgE of higher affinity than individuals who are just sensitized. T<sub>FH</sub>13 cells regulate the induction of anaphylactic IgE [30] by secreting cytokines, T<sub>FH</sub> cells guide the production of specific antibody isotypes during an immune response. The high-affinity IgE prevalent in allergy situations cannot be induced only by T<sub>FH</sub> cell-derived IL-4, although it is necessary for IgE synthesis. Additionally, IL-13, produced by a recently discovered T<sub>FH</sub>13 cell population, is needed.

Furthermore, the affinity of IgE could induce different patterns of signaling. As some studies have reported [23,31], a high-affinity IgE can yield a more robust activation of phospho-LAT1, increasing degranulation and cytokine production with greater recruitment of neutrophils at the site of inflammation. On the contrary, a low-affinity IgE can induce the activation of other molecules, such as phospho-LAT2 or phospho-Fgr, increasing the production of the chemokines such as CCL2, CCL3, and CCL4, that are monocyte or macrophage attracting factors. So, the affinity of IgE could switch the cellular response by molecular signals [23]. Another interesting factor to consider is epitope diversity related to the affinity of sIgE. Some studies reported a higher epitope diversity with a high-affinity sIgE in allergic patients compared to those with tolerance, correlating with the severity of allergic reactions [47–49]. In our study, we show that sera from anaphylaxis patients induce a more robust activation of phospho-LAT1 in LAD2 cells and a higher amount of IL-8 and GM-CSF in CD34<sup>+</sup>-derived MCs from patients and LAD2 cells, suggesting a more pro-inflammatory pattern. Otherwise, sera from sensitized patients induce a higher amount of TGF- $\beta$  and CCL2 in CD34<sup>+</sup>-derived MCs and LAD2 cells, indicating a more protective pattern. TGF- $\beta$  was reported to suppress mast cell activity and to inhibit mast cell Fc $\epsilon$ RI expression in mice [50,51]. These results reinforce that differences in IgE affinity and increased epitope diversity lead to different cell activation programs that may lead to anaphylaxis in a high-affinity context.

Nevertheless, the MC contribution (cellular component) may also play a role in severity [52]. A mutation in KARS, which encodes the Lysine tRNA synthetase (LysRS), increases microphthalmia-associated transcription factor (MITF) activity has been associated to severe anaphylaxis [39]. Controversely, hyper-IgE syndrome patients (AD-HIES) with dominant negative *STAT3* mutations is protective [53]. In addition, an increased risk of severe anaphylaxis has been linked to hereditary variations in the copy number of the *TPSAB1* gene, which encodes tryptase [54]. In our study, serum tryptase

values below 8  $\mu\text{g/L}$  in all MC donors made the presence of hereditary alpha tryptasaemia unlikely.

The tIgE/sIgE ratio may be necessary for diagnostic purposes since the clinical relevance of the sIgE level depends on its fractional relation to tIgE when determining the receptor occupancy rate of effector cells. Furthermore, the measurement of allergen-specific sIgG<sub>4</sub> could be of additional value to indicate the development of tolerance in FA patients [55–57]. Indeed, serum IgG<sub>4</sub> levels are higher among asymptomatic atopic patients [58]; thus, the IgG<sub>4</sub>/IgE ratio is higher in nonatopic and asymptomatic atopic than in allergic patients [59,60]. We have observed that anaphylaxis patients had significantly lower tIgE/sIgE and sIgG<sub>4</sub>/sIgE, indicating higher relative sIgE values. However, the evidence regarding the utility of these ratios is still limited [61].

The results are significant and consistent, considering that the number of patients is not elevated, and allows us to conclude that mast cell activation profile analysis may discriminate patients at risk of developing anaphylaxis from those merely sensitized, helping risk stratification before an oral food challenge. Indeed, identifying a larger T<sub>FH13</sub> population in peripheral blood may also aid this decision-making process.

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**CONFLICT OF INTEREST**

MPC received honoraria for presentations from ThermoFisher Scientific and LETI Pharma SLU. JB received honoraria for consulting fees (advisory role) from Bial and Novartis; payment lectures from Hal Allergy, LETI Pharma, Menarini, Novartis and Thermofisher Scientific.

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## TABLES

**Table 1.** Subjects selected for preparing “pooled sera”.

Pool	Subject ID	Total IgE (KU <sub>A</sub> /L)	Pru p 3 IgE (KU <sub>A</sub> /L)	Ratio tIgE:slgE	Pru p 3 IgG4 (mg <sub>A</sub> /L)	Ratio slgG4/slge	Symptomatology
Anaphylaxis	1	48.2	11.3	4.26	ND	ND	Anaphylaxis (urticaria, bronchoespasm) with pomegranate
	2	117	10.7	10.93	ND	ND	Anaphylaxis (urticaria, bronchoespasm, hipotension, loss of conciousness) with tomato and cofactor (NSAID)
	3	136	61	2.22	ND	ND	Anaphylaxis (urticaria, vomiting, bronchoespasm) with apple and cofactor (physical exercise). OAS with peanut. Contact urticaria with peach.
	4	177	9.46	18.71	ND	ND	Anaphylaxis (urticaria, hipotension, bronchoespasm) with peach, hazelnut and peanut. Contact urticaria with peach.
	5	139	7.96	17.46	ND	ND	Anaphylaxis (urticaria, diarrhea, bronchoespasm) with peanut. Urticaria with corn, peanut and hazelnut. OAS with peach, tomato and lettuce.
	6	96.6	5.8	16.65	ND	ND	Anaphylaxis (urticaria, bronchoespasm) with walnut and cofactor (alcohol). Contact urticaria with peach. OAS with walnut and hazelnut
	7	132	16.5	8.00	ND	ND	OAS with apple, hazelnut. Urticaria and angioedema with tomato. Anaphylaxis (angioedema, diarrhea, bronchoespasm, hipotensión) with walnut and cofactor (physical exercise)
	8	159	17.7	8.98	ND	ND	Gastrointestinal symptoms with lettuce, tomato, green beans. OAS with hazelnut, peanut and walnut. Anaphylaxis (urticaria, angiedema, hipotension) with walnut with cofactor (NSAID)
Pool	ND	19.80	ND	2.31	8.59		
Sensitized	9	229	0.88	260.22	ND	ND	Asymptomatic sensitization
	10	160	1.25	128.00	ND	ND	Asymptomatic sensitization
	11	228	1.64	139.02	ND	ND	Asymptomatic sensitization
	12	316	1.22	259.01	ND	ND	Asymptomatic sensitization
Pool	ND	0.98	ND	0.46	2.13		
Healthy	13	ND	<0.10	ND	ND	ND	Healthy individuals
	14	ND	<0.10	ND	ND	ND	Healthy individuals
	15	ND	<0.10	ND	ND	ND	Healthy individuals
	16	ND	<0.10	ND	ND	ND	Healthy individuals
	17	ND	<0.10	ND	ND	ND	Healthy individuals
Pool	ND	<0.10	ND	0.09	0.56		

Characteristics of subjects selected for pooled sera. All healthy volunteers had Prup 3 sIgE <0.10 KU<sub>A</sub>/L. ND= Not determined. OAS= Oral Allergy Syndrome. NSAID= non-steroidal anti-inflammatory drugs.

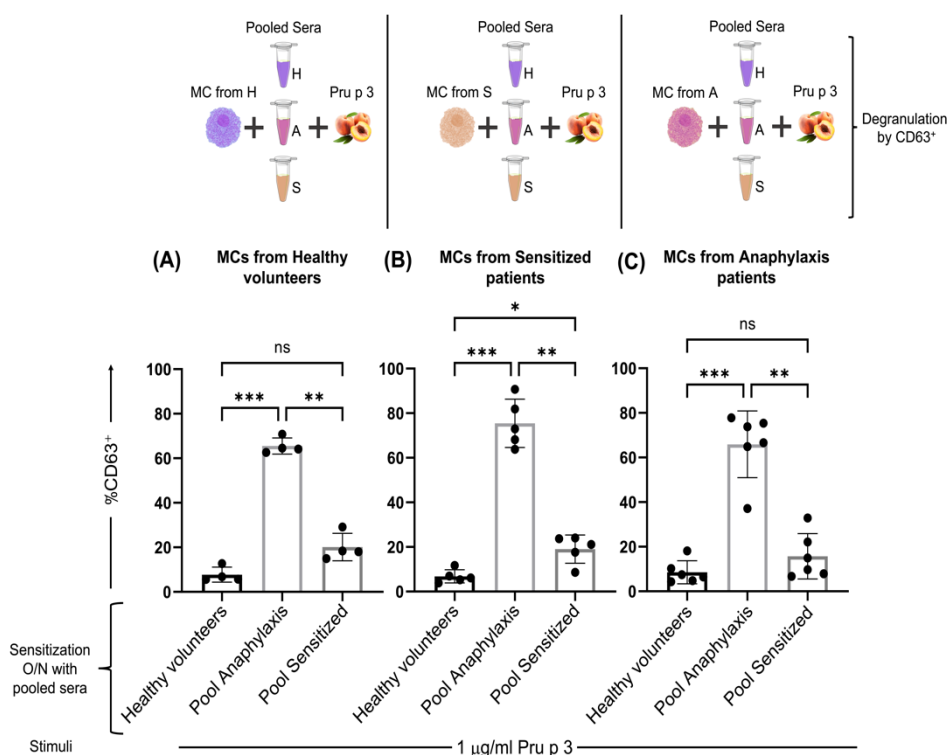
**Table 2.** Subjects selected for CD34<sup>+</sup>-derived MCs and T<sub>FH</sub>13 detection.

Subject ID	Group	Gender	Age	Prup 3 IgE (KUA/L)	Tryptase (µg/L)	Symptomatology
1	Anaphylaxis	Male	30	24.7	2.2	OAS with sunflower seed, pistachio, almond, peanut, walnut, hazelnut, lettuce, lentil, apple, grapefruit, avocado, banana, orange. Anaphylaxis (urticaria, hipotension, bronchoespasm) without cofactor with nuts, sunflower seeds, peach juice, vegetable mix.
2	Anaphylaxis	Male	50	1.05	2.3	Urticaria and angioedema with seeds. Anaphylaxis (urticaria, angioedema and bronchoespasm) with apple. Contact urticaria with peach. OAS with several nuts (hazelnut, walnut, peanut).
3	Anaphylaxis	Male	55	7.96	4.5	Anaphylaxis (urticaria, abdominal pain, vomitin, bronchoespasm) without cofactor with peach, hazelnut, and walnut.
4	Anaphylaxis	Female	42	8.76	ND	Urticaria with ingestion of mixed vegetables, apple. Gastrointestinal symptoms with green beans. Anaphylaxis (urticaria, angioedema, bronchoespasm) with almonds and cofactor (NSAID).
5	Anaphylaxis	Female	32	13.00	2.4	Anaphylaxis (urticaria, bronchoespasm, hipotension) without cofactor with peach and walnut.
6	Anaphylaxis	Male	56	16.50	5.4	OAS with ingestion of peanut, corn, walnut. Gastrointestinal symptoms with mixed vegetables and peach. Urticaria and angioedema with peach and nuts. Anaphylactic shock (urticaria, lingual angioedema, hipotension, loss of conciousness) with nectarine with cofactor (physical exercise).
7	Sensitized	Male	63	0.43	5.7	Asymptomatic sensitization
8	Sensitized	Female	46	41.20	3.9	Asymptomatic sensitization
9	Sensitized	Female	37	28.70	6.3	Asymptomatic sensitization
10	Sensitized	Male	48	0.66	5.2	Asymptomatic sensitization
11	Sensitized	Female	74	1.25	4.4	Asymptomatic sensitization
12	Healthy	Male	30	0.03	ND	Healthy individuals
13	Healthy	Female	22	0.03	ND	Healthy individuals
14	Healthy	Male	28	0.02	ND	Healthy individuals
15	Healthy	Male	35	0.03	ND	Healthy individuals

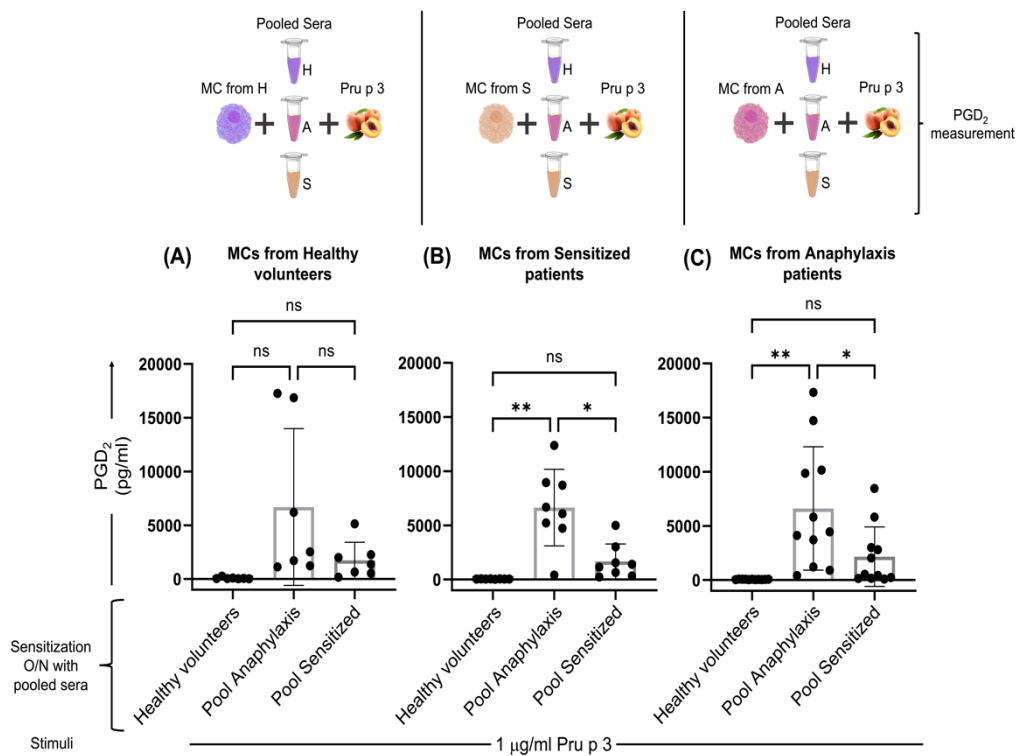
Characteristics of subjects selected for CD34<sup>+</sup>-derived MCs and T<sub>FH</sub>13 detection. ND= Not determined. OAS= Oral Allergy Syndrome. NSAID= non-steroidal anti-inflammatory drugs.

## FIGURES

**Figure 1. Anaphylaxis sera induce greater MC degranulation.** Degranulation measured by CD63 expression was performed with **A)** MCs from healthy volunteers (n=4), **B)** MCs from sensitized patients (n=5), and **C)** MCs from anaphylaxis patients (n=6). MCs were sensitized overnight with pooled sera and stimulated with 1  $\mu\text{g/ml}$  of Pru p 3. Results are expressed as mean  $\pm$  SD.  $P < 0.05$  was considered significant. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . MC=Mast cells; H=Healthy volunteers; A=Anaphylaxis; S=Sensitized.

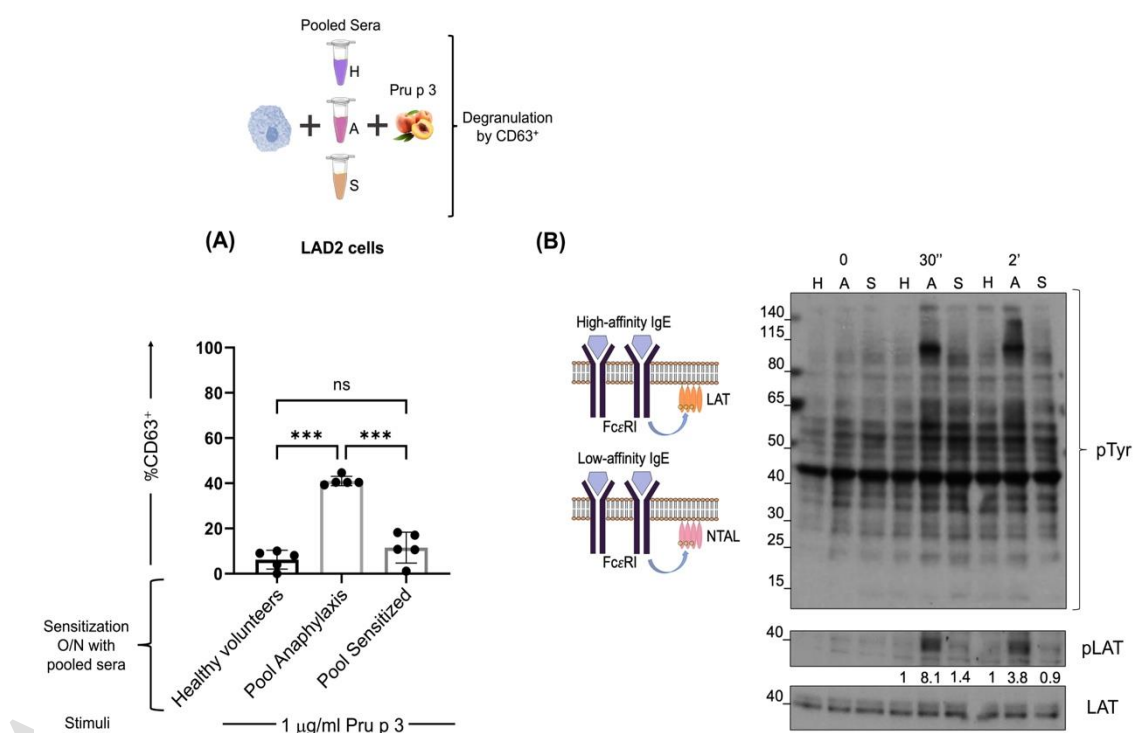


**Figure 2. Anaphylaxis sera induce higher PGD<sub>2</sub> secretion.** PGD<sub>2</sub> secretion was performed with **A**) MCs from healthy volunteers (n=4), **B**) MCs from sensitized patients (n=5), and **C**) MCs from anaphylaxis patients (n=6). PGD<sub>2</sub> was measured in post-activation supernatant. Results are expressed as mean  $\pm$  SD.  $P < 0.05$  was considered significant. \* $P < 0.05$ ; \*\* $P < 0.01$ . MC=Mast cells; H=Healthy volunteers; A=Anaphylaxis; S=Sensitized.

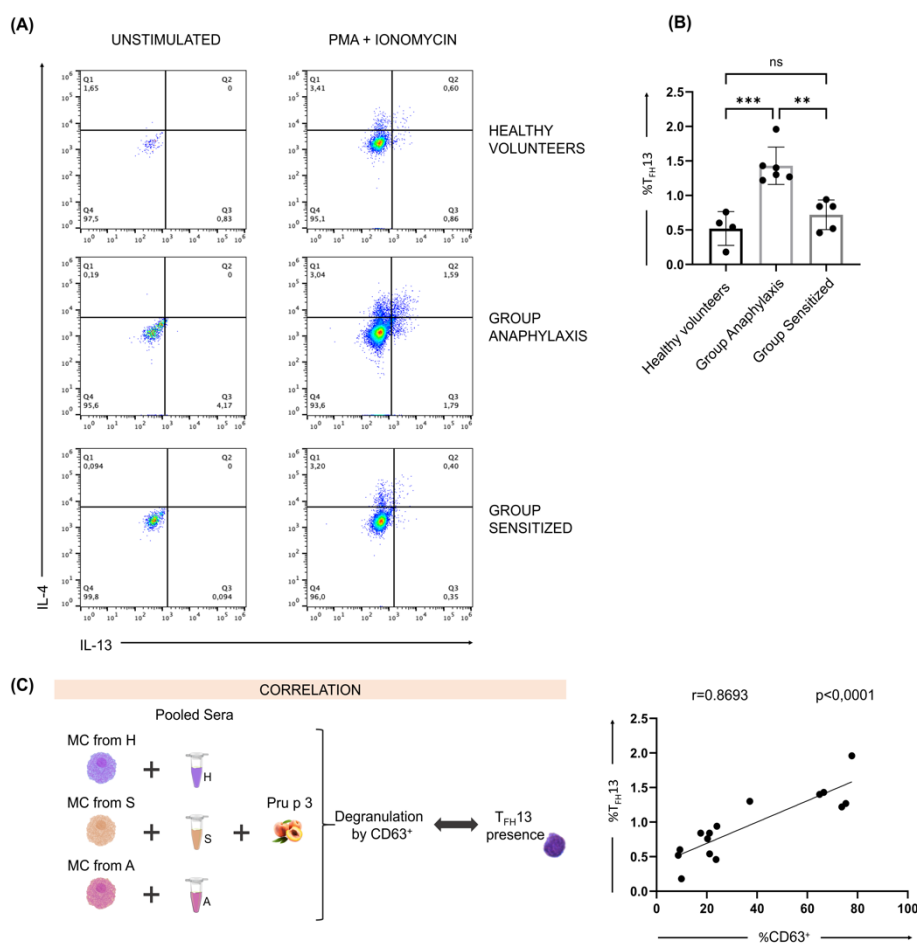




**Figure 3. Anaphylaxis sera induce higher LAD2 activation. A)** Degranulation measured by CD63 was performed with LAD2 cells sensitized overnight with pooled sera and stimulated with 1  $\mu\text{g/ml}$  Pru p 3 for 30 seconds and 2 minutes (n=5). Results are expressed as mean  $\pm$  SD.  $P < 0.05$  was considered significant. \* $P < 0.05$ ; \*\* $P < 0.01$ . **B)** Western blot of LAD2 cells sensitized overnight with pooled sera and stimulated with 2  $\mu\text{g/ml}$  Pru p 3. pTyr=phosphor-Tyrosine; pLAT=phospho-LAT; LAT=Total LAT; MC=Mast cells; H=Healthy volunteers; A=Anaphylaxis; S=Sensitized.



**Figure 4. T<sub>FH</sub>13 cells are more abundant in anaphylaxis patients** **A)** IL-4 and IL-13 intracellular staining in a healthy volunteer, an anaphylaxis patient, and a sensitized patient with or without PMA/ionomycin stimulation (Gated as in Figure S3). **B)** T<sub>FH</sub>13 cells in healthy volunteers (n=4), anaphylaxis (n=6), and sensitized patients (n=5). **C)** Correlation between degranulation and the percentage of T<sub>FH</sub>13 cells. Results are expressed as mean  $\pm$  SD. Significance was determined using one-way ANOVA with Tukey's multiple comparison analysis. Correlations were calculated by using Pearson R values.  $P < 0.05$  was considered significant. \*\*\* $P < 0.001$ ; \*\*\*\* $P < 0.0001$ . MC=Mast cells; H=Healthy volunteers; A=Anaphylaxis; S=Sensitized.



**Figure 5. Sera from anaphylaxis patients induce higher pro-inflammatory patterns.** Cytokine multiplex assay was performed in **A)** CD34<sup>+</sup>-derived MCs from anaphylaxis (n=6) and sensitized patients (n=5) and in **B)** LAD2 cells (n=3). Results are expressed as mean  $\pm$  SD. Significance was determined using a t-test with Welch's correction.  $P < 0.05$  was considered significant. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\*\* $P < 0.0001$ . MC=Mast cells; A=Anaphylaxis; S=Sensitized. IgE<sup>b</sup>=biotinylated human IgE; STV=Streptavidin.

