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# Basophils from allergic patients are neither hyper-responsive to activation signals nor hypo-responsive to inhibition signals

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**Contributions:** LC received blood samples, performed all the experimental work and generated all experimental data; KS and T-PB created and exploited the clinical data base; AC performed all statistical analyses and generated graphs used for figures; RB-S managed the cooperation between Institut Pasteur and *Danone Research*; MA hosted LC in the *Centre d'Immunologie Humaine* (CIH) he was the director of, provided the necessary logistics and enabled CIH personnel to be available for this project; EM took care of legal issues, obtained necessary permissions, established and managed reglementary documents; JL and M-TG took care of all medical issues, selected patients, obtained their informed consent, made groups and collected clinical data; MD conceived the project, supervised the experimental work and wrote the manuscript.

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**Conflicts of interest:** This work was performed within the frame of a partnership between Institut Pasteur and *Danone Research*. Lydie Cassard was financially supported by *Danone Research*; Katia Sperber is now a Clinical Research Associate/Data Manager at *Virbac*; Aurélie Cotillard was commissioned by *Soladis* to perform consulting for *Danone Research*; Raphaëlle Bourdet-Sicard was and still is a Senior Scientist at *Danone Research*; Matthew Albert is now a Principal Scientist at *Genentech*. Marc Daëron was a consultant for *Danone Research* twice, in 2014 and 2015.

## Abstract

**Background:** Basophil activation contributes to inflammatory reactions, especially in allergy. It is controlled, positively and negatively, by several mechanisms. High-affinity IgE receptors (FcεRI) generate a mixture of activation and inhibition signals upon aggregation, whose ratio depends on the concentration of allergen recognized by receptor-bound IgE. Low-affinity IgG receptors (FcγRIIA/B) generate inhibition signals when co-engaged with FcεRI by allergen-antibody immune complexes. Commensal and probiotic bacteria such as *L. paracasei* generate inhibition signals by still unclear mechanisms.

**Objective:** Investigate whether mechanisms that control, positively and negatively, basophil activation, which were unraveled and studied in basophils from normal donors, are functional in allergic patients.

**Methods:** FcεRI and FcγRIIA/B expression, FcεRI-dependent activation, FcεRI-dependent inhibition, and FcγRIIB-dependent inhibition were examined in blood basophils incubated overnight with *L. paracasei* or without, and challenged under 10 experimental conditions. Basophils from normal donors were compared with basophils from patients who consulted an allergology outpatient clinic over a period of 3 months with respiratory allergy, anaphylaxis antecedents, chronic urticaria, and/or atopic dermatitis.

**Results:** Patient basophils expressed neither more FcεRI nor less FcγRIIB than basophils from normal donors. They were neither hyper-reactive to positive regulation nor hypo-reactive to negative regulation, whatever the receptors or the mechanisms involved and whatever the allergic manifestations patients suffered from.

**Conclusion:** Regulatory mechanisms that control basophil activation are fully functional in allergic patients. Intrinsic defects in these mechanisms do not explain allergic manifestation. Based on these mechanisms, "immune checkpoint modifiers" can be developed as novel therapeutic tools for allergy.

## Capsule summary

Basophils from allergic patients are neither hyper-responsive to IgE-induced activation nor hypo-responsive to IgG- and/or lactobacilli-induced inhibition. Mechanisms that control basophil activation can therefore be used as therapeutic tools in allergic diseases.

## Key messages

1. Antibody-mediated FcεRI- and/or FcγRII-dependent positive and negative regulation, as well as lactobacilli-induced negative regulation are fully functional in basophils from allergic donors.
2. Intrinsic defects in mechanisms that control basophil activation do not account for the etiology of allergic diseases
3. The finding that no intrinsic abnormality dampens mechanisms that control basophil activation in allergic patients encourages "immune checkpoint modifiers" to be developed against allergies.

## Keywords

Anaphylaxis; Asthma; Atopic dermatitis; Basophil activation; Chronic urticaria; FcεRI; FcγRIIB; Lactobacilli; Negative regulation; Rhinitis.

## Abbreviations & acronyms used in the manuscript

APC, AlloPhycoCyanin  
 dsDNA, double-strain DesoxyriboNucleic Acid  
 EDTA, EthyleneDiamineTetraAcetic acid  
 Erk1/2, Extracellular signal-regulated kinases 1 & 2  
 F(ab')<sub>2</sub> fragment, IgG antibody the Fc portion of which was digested with pepsin  
 Fc, portion of antibody made of the 2-3 C-terminal constant heavy chain domains  
 FcεRI, High-affinity receptors for the Fc portion of IgE. Contain 2 ITAMs  
 FcγRIIA, Low-affinity receptors for the Fc portion of IgG. Contain 1 ITAM  
 FcγRIIB, Low-affinity receptors for the Fc portion of IgG. Contain 1 ITIM  
 FITC, Fluorescein IsoThioCyanate  
 IgE, Immunoglobulin E; hIgE, human IgE; rIgE, rat IgE  
 IgG, Immunoglobulin G  
 IL-4, InterLeukin 4  
 ITAM, Immunoreceptor Tyrosine-based Activation Motif  
 ITIM, Immunoreceptor Tyrosine-based Inhibition Motif  
 Jnk, c-Jun N-terminal Kinase  
 MAR, Mouse Anti-Rat immunoglobulin antibodies  
 p38 MAP kinase, Mitogen-activated protein kinase of 38 kDa  
 PBMCs, Peripheral Blood Mononuclear Cells  
 PBS, Phosphate-Buffered Saline  
 PE, PhycoErythrin  
 [PI(3,4,5)P<sub>3</sub>], Phosphatidylinositol 3,4,5-trisPhosphate  
 RAHE, Rabbit Anti-Human IgE antibodies  
 SHIP1, SH2 domain-containing Inositol 5-Phosphatase 1  
 Th1/Th2, Type 1/2 helper T lymphocytes

## INTRODUCTION

Basophils have been recognized to play critical roles in an increasing number of pathological conditions. Particularly well known is their contribution to allergy as the initiators of IgE-induced acute reactions. Basophils also account for late-phase (1) and chronic (2) allergic inflammation. They are a major source of IL-4 that promotes chemokine and cytokine secretion by type-2 innate lymphoid cells, leading to eosinophilic inflammation (3, 4). Autoreactive IgE anti-dsDNA antibodies associated with basophil activation were found in systemic lupus erythematosus patients (5), which were correlated with the severity of nephritis (6). Under normal conditions, however, basophils are under the control of a variety of regulatory mechanisms that may prevent the pathogenic consequences of their activation.

High-affinity IgE receptors (FcεRI) activate basophils when aggregated by a plurivalent allergen that binds to FcεRI-bound IgE antibodies. FcεRI aggregation, however, generates not only activation signals, but also inhibition signals, whose ratio depends on the degree of receptor aggregation, *i.e.* on antigen concentration. Positive signals are dominant over negative signals up to an optimal concentration, beyond which negative signals become dominant and abrogate basophil activation. This auto-inhibition of FcεRI signaling depends on the SH2 domain-containing inositol 5-phosphatase (SHIP1) (7). By hydrolyzing phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P<sub>3</sub>], which mediates the membrane recruitment of cytosolic signaling molecules with a pleckstrin homology domain (8), SHIP1 inhibits distal signaling events, among which Ca<sup>2+</sup> mobilization, the activation of Erk1/2, Jnk and p38 MAP kinases, and their biological consequences.

FcγRIIB-dependent negative regulation further controls mast cell (9) and basophil activation (10). As one million-fold more IgG than IgE (mg/ml vs ng/ml) circulate in the bloodstream, allergens are likely to interact with FcεRI-bound IgE as IgG immune complexes, rather than in isolation. Allergens therefore do not simply aggregate FcεRI; they co-aggregate FcεRI with low affinity IgG receptors expressed on the same cell. These include activating (FcγRIIA) and inhibitory (FcγRIIB) receptors. As human basophils express small amounts of FcγRIIA (10-fold less than blood monocytes), but high amounts of FcγRIIB (3-fold more than blood B cells), negative regulation is dominant when FcγRIIA and FcγRIIB are co-engaged by immune complexes on basophils from normal donors (10). Inhibition induced under these conditions also depends on SHIP1, high amounts of which are recruited by FcγRIIB. FcγRIIB indeed contains an Immunoreceptor Tyrosine-based Inhibition Motif (ITIM) which, when phosphorylated, has a high affinity for the SH2 domain of SHIP1 (11).

Commensal bacteria have increasingly been understood to play critical roles in the homeostasis that controls inflammatory processes, and IgE-dependent human basophil activation can be profoundly but reversibly inhibited when peripheral blood mononuclear cells (PBMCs) are exposed to specific strains of *L. paracasei* before challenge (12). Inhibition requires a direct contact between cells and bacteria, but the molecular mechanism accounting for inhibition was not elucidated.

These regulatory mechanisms have been increasingly regarded as potential therapeutic tools for controlling the effector phase of inflammation, especially in allergy. Allergen immunotherapy is currently thought to depend on the generation of regulatory T cells that promote a state of "tolerance" to allergen by dampening Th2 responses (13). It nevertheless induces high amounts of allergen-specific IgG antibodies that have long been believed to compete with specific IgE for antigen (14). IgG antibodies found in the serum of

patients treated by immunotherapy were recently shown to dampen basophil activation via a FcγRIIB-dependent mechanism (15, 16). The consumption of probiotic bacteria has been proposed as a prophylactic measure to prevent either the onset of allergies in normal individuals or the outcome of allergic symptoms in allergic patients (17). Bispecific molecules capable of co-ligating FcγRIIB with either FcεRI (18) or FcεRI-bound IgE (19, 20) have been generated as potential new treatments of allergies, and genetically engineered IgG antibodies have been produced, among which anti-IgE antibodies, whose affinity for FcγRIIB is several hundred-fold higher than that of native antibodies (21, 22).

The efficacy of these therapeutic approaches implies that the regulatory mechanisms they lie on are functional in allergic patients. These mechanisms were indeed described and studied in basophils from normal donors. They were not examined in patients. If, whatever the reason, one or several of these mechanisms are deficient in one or several allergic conditions, therapeutic tools may be disqualified for one or several allergic diseases. On the other hand, finding that negative regulation is impaired in some or all allergic patients, whatever the mechanism, may provide a clue to understanding the etiology of allergies. What makes patients allergic and/or what prevents non-allergic individuals from being allergic is not known, and one possibility is that negative regulation is operational and protective in non-allergic individuals, but impaired in allergic patients.

We therefore examined FcεRI-dependent positive and negative regulation, FcγRIIB-dependent negative regulation, and *L. paracasei*-induced inhibition in basophils from patients who are routinely taken care of in an allergology outpatient clinic. We found that all regulatory mechanisms examined were fully functional in patients whatever their pathological conditions.

## PATIENTS AND EXPERIMENTAL PROCEDURES

### 1. Patients and normal donors

A total of 77 patients (57 females and 20 males), 18-77 year-old (mean 44), were enrolled in the study over a 3-month period (from October 22, 2013 to January 29, 2014). Inclusion criteria were as follows: Patients older than 18, suffering from an allergic disease, to whom a blood test had been prescribed, who agreed to be enrolled in the study by giving their informed consent. Exclusion criteria were as follows: Patients unable to give their consent, patients with evolutive neoplasia, infectious or inflammatory disease, patients having received glucocorticoid or anti-histamine medication less than one week earlier, and patients with immunosuppressive treatment, whatever the reason. Patients were recruited as part of the Institut Pasteur 2013-13 clinical study with ethical approval from the *Comité de Protection des Personnes Ile-de-France II*.

Diagnostics were based of clinical symptoms, skin tests and biological measurements. Patients were classified by clinicians into 5 groups as a function of diagnosis (Fig. 1A): *Group A* (16 patients), *respiratory allergy*. Patients sensitized to aeroallergens and/or with a personal history of atopy suffered from recurrent spasmodic coryza and/or asthma with bronchial hyper-reactivity defined by a 20% FEV1 increase after inhalation of 200 µg salbutamol as specified in (23, 24); *Group B* (16 patients), *anaphylaxis antecedents*. Patients displayed generalized urticarial, respiratory obstruction, severe tachycardia with blood pressure drop and/or loss of consciousness occurring immediately after hymenoptera sting, food ingestion or drug intake corresponding to grades 2-3 as defined in (25); *Group C* (20 patients), *chronic urticaria*. Patients displayed chronic urticaria symptoms as specified in (26) for more than 6 weeks; *Group D* (16 patients), *atopic dermatitis*. Patients suffered from a long lasting dermatitis with childhood onset and persistence in adulthood, affecting head and neck and for some patients the popliteal and the antecubital fossa as specified in (27); *Group A+* (9 patients), *respiratory allergy plus one other allergic condition* (1 anaphylaxis, 2 chronic urticarias, 5 food allergies, 1 Quincke edema). A sixth group (*Group N*) consisted of 23 anonymous normal blood donors from the *Etablissement Français du Sang* who also provided written informed consent for their blood to be included in the research program.

In practice, one 5-10-ml blood sample was collected on EDTA at the occasion of a blood test that had been prescribed to investigate the clinical condition of the patient, independently of the study. One blood sample only from each patient was examined. Blood from 1-5 patients were tested every day as they came, together with one blood sample drawn on the same day from a normal donor, which was included as a positive control in every experiment. All blood samples were examined with the same protocol under the same conditions. Data from all blood samples were included in the analysis except 1) 1 group A- and 3 group C-samples that contained not enough basophils for being analyzed, 2) 2 group N-, 3 group A-, 2 group A+, 6 group B-, 10 group C-, and 3 group D-samples in which basophils failed to be activated upon FcεRI aggregation by one or both anti-IgE reagents used. Data from 2 donors were also missing, due to a technical error. Noticeably, for the above reasons, 6 group C-samples only could be exploited out of 20. Blood samples were numbered by chronological order. Experiments were performed blindly as only clinical doctors knew diagnosis. No diagnostic information was disclosed until experiments with blood samples from all subjects were completed and all data filed and analyzed. Conversely, experimental results were not disclosed to clinicians until the investigation was completed.

## 2. Experimental procedures

**Cells.** PBMCs, isolated from peripheral blood by centrifugation through Ficoll-Hypaque, were resuspended at  $1 \times 10^6$  cells/ml in culture medium (RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, 1% HEPES buffer) and sensitized by an overnight incubation at 37°C with 3 µg/ml myeloma rat IgE IR162 (rIgE) (IMEX, Université de Louvain, Bruxelles, Belgium). PBMCs were placed at 37°C for overnight incubation within 1 hr after blood was drawn. They were then submitted to the 10 experimental conditions shown in Table I. All 10 conditions were applied to PBMCs from the same donor in the same experiment.

**Bacteria.** *L. paracasei* were the same strain (CNCM I-1518) used in our previous work (12). They were cultured in Mann-Rogosa-Sharpe broth at 37°C for 48h, washed, resuspended in PBS and adjusted to an  $OD_{600} = 2$ . Ten microliters *L. paracasei* were added to 90 µl PBMC, together with rIgE. Cells were therefore incubated overnight with bacteria.

**Challenge.** Cells were washed extensively and stimulated for 20 min. at 37°C with equimolar concentrations of the following 4 reagents: 1) 15 µg/ml rabbit IgG anti-human IgE (RAHE IgG, Dako-Cytomation, Trappes, France); 2) 10 µg/ml F(ab')<sub>2</sub> fragments obtained by peptic digestion of the same antibodies (RAHE F(ab')<sub>2</sub>); 3) 15 µg/ml mouse IgG anti-rat Ig antibodies (MAR IgG) (Jackson ImmunoResearch, West Grove, PA); 4) 10 µg/ml F(ab')<sub>2</sub> fragments of the same antibodies (MAR F(ab')<sub>2</sub>, Jackson ImmunoResearch). Basophils were identified as FcεRI<sup>+</sup> CD203c<sup>+</sup> cells as described (28), and basophil activation was monitored by CD203c up-regulation as described (10).

**Immunofluorescence analysis.** Cells were stained with phycoerythrin (PE)-conjugated anti-CD203c antibodies (Immunotech, Marseille, France), allophycocyanin (APC)-conjugated anti-FcεRIα antibodies (eBioscience, San Diego, CA), fluorescein isothiocyanate (FITC)-conjugated anti-human IgE antibodies (Sigma Aldrich, St Louis, MO) FITC-conjugated anti-FcγRIIA (IV.3) (Stemcell technologies, Vancouver, Canada) or Vioblue-anti-FcγRIIA+B (2E1) antibodies (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) and corresponding isotype controls. All incubations were done at 0°C and, when applicable, after challenge. Basophils were identified as FcεRIα<sup>+</sup> CD203c<sup>+</sup> cells. Basophil viability was monitored with Topro-3 (Life Technologies). Fluorescence was assessed by flow cytometry.

## 3. Statistical analysis

Statistical analyses were performed using the SAS 9.3 and R 3.3.0 softwares. Either raw values or variations between two conditions were analyzed. Wilcoxon signed-rank tests were used to compare two conditions in normal donors. Kruskal-Wallis tests were used to compare data between independent groups and Friedman tests were used to compare data between paired groups. In addition, a Bonferroni-Holm adjustment was performed for post-hoc comparisons. Results with  $p < 0.05$  were considered significant.

## RESULTS

FcεRI and FcγRIIA/B expression, anti-IgE-induced activation, FcεRI auto-inhibition and FcγRIIB-dependent inhibition were compared in basophils from normal blood donors (Group N) and in basophils from patients with respiratory allergy (Group A), anaphylaxis antecedents (Group B), chronic urticaria (Group C), atopic dermatitis (Group D), or respiratory allergy plus one other allergic condition (Group A<sup>+</sup>). The effects of *L. paracasei* were examined on FcεRI and FcγRIIA/B expression, on anti-IgE-induced activation, on FcεRI auto-inhibition and on FcγRIIB-dependent inhibition in basophils from the same 6 groups of donors.

### 1. Basal parameters

As expected (29) and as observed previously (28), plasma IgE levels were significantly higher in atopic dermatitis patients (Group D) than in other allergic patients (Fig. 1B). Consequently, basophils from Group D patients displayed more FcεRI-bound human IgE (hIgE) and expressed more FcεRI than basophils from normal donors (Fig. 2A). No statistically significant difference in basophil numbers was observed between the 5 groups of patients (Fig. 1C). No statistically significant difference in CD203c expression was observed between basophils from the 5 groups of patients and from normal donors (Fig. 2A).

Basophils from all 5 groups of patients expressed comparable amounts of FcγRII as basophils from normal donors, when assessed either with FcγRIIA-specific or with FcγRIIA+B-specific mAbs (Fig. 3A). Because the expression of FcγRIIA is very low, whereas the expression of FcγRIIB is very high in human basophils (10), FcγRIIA+B-specific mAbs reveal essentially FcγRIIB.

### 2. Effect of *L. paracasei* on resting cells

An overnight incubation of PBMCs with *L. paracasei* altered several parameters on resting basophils from normal donors. CD203c expression was increased almost 2 fold, whereas FcεRI-bound hIgE was decreased. Neither FcεRI expression (in spite of a statistically significant difference) (Fig. 2B) nor FcγRIIA expression were detectably altered (Fig. 3B), whereas FcγRIIA+B expression was decreased by one third (Fig. 3B).

*L. paracasei* similarly affected basophils from normal donors and basophils from allergic patients. However, *L. paracasei*-induced differences that were statistically different in basophils from normal donors were not statistically different in basophils from allergic patients (Figs. 2B and 3B). Also, both the elevated FcεRI expression and FcεRI-bound IgE levels seen in basophils from atopic dermatitis patients decreased to levels that were not statistically different from levels seen in basophils from normal donors and from other patients, following exposure to *L. paracasei* (Fig. 2B).

### 3. FcεRI-dependent basophil activation

When recognizing membrane-bound endogenous hIgE, RAHE F(ab')<sub>2</sub> fragments aggregate FcεRI and trigger basophil activation that can be monitored by assessing the up-regulation of CD203c by flow cytometry (Table I, condition 1). RAHE F(ab')<sub>2</sub> indeed increased CD203c expression 3 fold in basophils from normal donors. A similar 2-3-fold up-regulation of CD203c was induced under the same conditions in basophils from the 5 groups of patients (Fig. 4A). One should remind, however, that basophils from 10 patients of group-C which

could be analyzed did not respond to RAHE F(ab')<sub>2</sub>. Basophils from the remaining samples responded similarly as basophils from normal donors and the slight differences in CD203c up-regulation seen in basophils from the various groups of patients were not statistically significant.

All FcεRI are not occupied by endogenous hIgE. Cells were therefore passively sensitized with rIgE. Human FcεRI can indeed bind rIgE with a similarly high affinity as hIgE (30). When recognizing receptor-bound rIgE, MAR F(ab')<sub>2</sub> fragments aggregate FcεRI and trigger basophil activation (Table I, condition 5). MAR antibodies do not cross-react with hIgE (10). MAR F(ab')<sub>2</sub> increased CD203c expression 2-3 fold in basophils from normal donors, as well as in basophils from the 5 groups of patients (Fig. 4B). Basophils from the group C patients that were not activated by RAHE F(ab')<sub>2</sub> were not activated either by MAR F(ab')<sub>2</sub>. Differences in CD203c up-regulation seen in basophils from the various groups of patients were not statistically significant.

#### 4. FcγRIIB-dependent inhibition of FcεRI-dependent basophil activation

When recognizing receptor-bound hIgE, intact RAHE IgG antibodies co-aggregate FcεRI *via* their Fab portions and FcγRIIB+A *via* their Fc portion on the same cells (Table I, condition 2). Under this condition, FcγRIIB inhibit FcεRI-dependent activation (10). Inhibition can be monitored by comparing CD203c up-regulation induced under condition (2) with CD203c up-regulation induced under condition (1). RAHE IgG induced a 40-60% inhibition in normal donors. Inhibitions of comparable magnitudes were observed in basophils from the 5 groups of patients (Fig. 5A). Differences between normal donors and patients of the 5 groups were not statistically significant.

When recognizing receptor-bound rIgE, intact MAR IgG antibodies co-aggregate FcεRI *via* their Fab portions and FcγRIIB+A *via* their Fc portion on the same cell (Table I, condition 7), and FcγRIIB inhibit FcεRI-dependent activation. Inhibition was monitored by comparing CD203c up-regulation induced under condition (7) with CD203c up-regulation induced under condition (5). MAR IgG induced a ±70% inhibition in basophils from normal donors, and inhibitions of comparable magnitudes in basophils from the 5 groups of patients (Fig. 6A).

#### 5. *L. paracasei*-induced inhibition of FcεRI-dependent basophil activation

An overnight incubation of PBMCs with *L. paracasei* strain CNCM I-1518 inhibits the subsequent IgE-mediated FcεRI-dependent activation of human basophils (12). The inhibitory effect of *L. paracasei* was examined on basophil activation induced by RAHE F(ab')<sub>2</sub> (Table I, condition 2) or by MAR F(ab')<sub>2</sub> (Table I, condition 6). Inhibition was assessed by comparing conditions (2) and (1) and condition (5) and (6). *L. paracasei* induced a more than 80 % inhibition of basophil activation, whether induced by RAHE F(ab')<sub>2</sub> (Fig. 5B) or by MAR F(ab')<sub>2</sub> (Fig. 6B). *L. paracasei* induced inhibitions of comparable magnitudes in basophils from normal donors and from the 5 groups of patients whether challenged with RAHE F(ab')<sub>2</sub> or with MAR F(ab')<sub>2</sub>.

#### 6. MAR F(ab')<sub>2</sub>-induced FcεRI auto-inhibition

As expected, CD203c up-regulation displayed a bell-shaped curve when basophils passively sensitized with rIgE were challenged with increasing concentrations of MAR F(ab')<sub>2</sub> (not shown). An excess of ligand indeed induces a dose-dependent inhibition of cell activation that depends on the lipid phosphatase SHIP1 (7). This FcεRI auto-inhibition was assessed

by comparing CD203c up-regulation induced by an optimal concentration of 10 $\mu$ g/ml (1X) MAR F(ab')<sub>2</sub> (Table I, condition 1) and CD203c up-regulation induced by two supra-optimal concentrations (9x and 18X) of MAR F(ab')<sub>2</sub> (Table I, condition 9). 9X MAR F(ab')<sub>2</sub> induced a  $\pm$  50% inhibition in basophils from normal donors (Fig. 6D), and 18X MAR F(ab')<sub>2</sub> an inhibition of  $\pm$  60% (Fig. 6E). Fc $\epsilon$ RI auto-inhibition was of comparable magnitudes in basophils from normal donors and in basophils from patients. One notices that the non-statistically significant differences between the 5 groups of patients observed with 9X MAR F(ab')<sub>2</sub> were blunted with 18X MAR F(ab')<sub>2</sub>.

## 7. Fc $\gamma$ RIIB-dependent inhibition or Fc $\epsilon$ RI auto-inhibition inhibition + *L. casei*-induced inhibition

Unlike the well-established SHIP1-mediated mechanism of Fc $\epsilon$ RI auto-inhibition and Fc $\gamma$ RIIB-dependent inhibition, the molecular mechanism by which *L. paracasei* inhibit basophil activation is poorly known. We wondered whether *L. paracasei*-induced inhibition would compete with SHIP1-mediated inhibition. PBMC that had been exposed to *L. paracasei* were challenged with RAHE IgG (Table I, condition 4), MAR IgG (Table I, condition 8) or supra-optimal concentrations of MAR F(ab')<sub>2</sub> (Table I, condition 10). Inhibitions of CD203c up-regulation observed under these conditions were compared with inhibitions induced by the same ligands in cells not exposed to *L. paracasei* (Table I, conditions 3, 7 & 9, respectively) and with inhibitions induced by *L. paracasei* alone (Table I, conditions 2 & 6).

(*L. paracasei* + RAHE IgG)-induced inhibition of normal basophil activation (Fig. 5C) was much deeper than inhibition induced by RAHE IgG alone (Fig. 5A) and slightly deeper than inhibition induced by *L. paracasei* alone (Fig. 5B). (*L. paracasei* + RAHE IgG)-induced inhibitions were similarly deeper than inhibition induced by *L. paracasei* or RAHE IgG alone in basophils from patients and in basophils from normal donors, except in Group B and D where inhibition induced by *L. paracasei* + RAHE IgG (Fig. 5C) was similar as inhibition induced by *L. paracasei* alone (Fig. 5B). Differences between normal donors and the 5 groups of patients, however, were not statistically significant.

(*L. paracasei* + MAR IgG)-induced inhibition of normal basophil activation (Fig. 6C) was also much deeper than inhibition induced by MAR IgG alone (Fig. 6A) or by *L. paracasei* alone (Fig. 6B). (*L. paracasei* + MAR IgG)-induced inhibitions were similarly deeper than inhibition induced by *L. paracasei* or MAR IgG alone in basophils from patients and in basophils from normal donors.

Inhibitions of normal basophil activation induced by *L. paracasei* and a supra-optimal concentration of MAR F(ab')<sub>2</sub> (Fig. 6F & 6G) were deeper than inhibition induced by an excess of MAR F(ab')<sub>2</sub> alone (Fig. 6D & 6E), but of the same magnitude as inhibition induced by *L. paracasei* alone (Fig. 6B). A similar additive effect was observed in basophils from the 5 groups of patients.

## DISCUSSION

Our investigation documents the functional state of blood basophils in a sample of 77 patients who consulted an allergology outpatient clinic over a period of 3 autumn-winter months. It was focused on mechanisms known to regulate, positively or negatively, the activation of basophils from normal donors. The unexpected overall result is that, compared with basophils from normal donors, patient basophils were neither hyper-reactive to positive regulation nor hypo-reactive to negative regulation, whatever the allergic manifestations they suffered from. This result has important consequences on our understanding of the etiology of allergies and on new therapeutic strategies that are being developed.

First of all, we noticed both expected and unexpected abnormalities in two groups of patients. As expected, basophils from atopic dermatitis patients (group D) expressed more FcεRI and displayed more FcεRI-bound hIgE than basophils from normal donors or from other patients. These differences were correlated with increased plasma IgE levels that are classically associated with atopic dermatitis (29). It is well-known that FcεRI expression is up-regulated by IgE (31) which slows-down the intracellular degradation of receptors occupied by IgE (32). Unexpected basophil abnormalities were observed in some, but not all chronic urticaria patients (group C). Three among the 19 patients included in this group (one of the 20 group-C samples could not be processed for irrelevant technical reasons) had basophil numbers too low for being studied, and the basophils of 10 others failed to respond to FcεRI aggregation, whether by RAHE or MAR F(ab')<sub>2</sub> fragments. The remaining 6 group-C patients had similar numbers of basophils, which responded similarly as normal donor basophils to FcεRI aggregation. Group C may therefore contain two subgroups of patients, 68% of which (13/19) having low basophil numbers or hypo-reactive basophils, and 32% (6/19) having normal numbers of normally reactive basophils. Chronic urticaria is a nosologically heterogeneous ensemble of diseases some of which are autoimmune. Autoantibodies against the α subunit of FcεRI are indeed found in the serum of 30-60% chronic urticaria patients (33, 34), which are thought to trigger basophil and/or mast cell activation responsible for clinical manifestations. Whether such autoantibodies were present in the serum of patients with abnormal basophil counts or functions is not known. Both low basophil numbers or hypo-reactive basophils could result from a chronic stimulation by anti-FcεRI autoantibodies. Besides, an unknown factor, present in the serum of active chronic idiopathic urticaria patients, was recently found, which inhibits FcεRI-dependent histamine release by basophils (35).

Remarkably, FcεRI expression and functions were unaltered in basophils from all other patients. Basophils from all patients except atopic dermatitis patients expressed comparable amounts of FcεRI as basophils from normal donors. Basophils from all patients except half of chronic urticaria patients, but including atopic dermatitis patients with an increased FcεRI expression, were similarly activated as basophils from normal donors by an optimal concentration of F(ab')<sub>2</sub> anti-IgE, whether *via* endogenous hIgE or *via* exogenous rIgE. The activation of basophils from all patients, including hypo-responsive basophils from half of chronic urticaria patients, was similarly inhibited by an excess of MAR F(ab')<sub>2</sub> fragments as basophils from normal donors. Basophils from allergic patients therefore did not over-express FcεRI, they were not over-responsive to FcεRI aggregation-triggered activation signals, and they were not hypo-responsive to FcεRI hyper-aggregation-triggered inhibition signals.

Likewise, FcγRIIA and FcγRIIB expression and functions were unaltered in basophils from all patients. FcγRIIA expression was comparable on basophils from patients as on basophils

from normal donors. Whether the activating properties of this ITAM-containing receptor were as modest as previously observed in basophils from normal donors (10) was not examined in basophils from patients. FcγRIIA+B expression (*i.e.* due to the markedly unbalanced expression of the two receptors, primarily FcγRIIB expression) was comparable on basophils from patients and on basophils from normal donors. Whether assessed with RAHE IgG or with MAR IgG, FcγRIIB-dependent negative regulation of FcεRI-dependent activation was similar in basophils from patients of all 5 groups as in basophils from normal donors. Basophils from allergic patients therefore did not express more FcγRIIA or less FcγRIIB and their activation was not less efficiently inhibited by the coaggregation of FcεRI with FcγRIIA+B than that of normal basophils.

*L. paracasei* similarly affected basophils from all groups of patients and basophils from normal donors. Basal CD203c expression was similarly increased, while FcεRI-bound hIgE and FcγRIIB expression were similarly decreased; FcεRI and FcγRIIA were similarly not changed. Noticeably, *L. paracasei* decreased both augmented FcεRI expression and FcεRI-bound hIgE levels back to normal in basophils from atopic dermatitis. *L. paracasei* inhibited similarly FcεRI-dependent activation in basophils from all patients and from normal donors, whether induced by RAHE F(ab')<sub>2</sub> or by MAR F(ab')<sub>2</sub>. *L. paracasei*-induced inhibition was additive with FcεRI auto-inhibition induced by an excess of ligand not only in basophils from normal donors, which had not been examined before, but also in basophils from all 5 groups of patients. Likewise, *L. paracasei*-induced inhibition was additive with FcγRIIB-dependent inhibition both in basophils from normal donors and in basophils from patients, in spite of the decreased FcγRIIB expression induced by *L. paracasei*. *L. paracasei* therefore inhibited as efficiently FcεRI-dependent activation in basophils from allergic patients and in basophils from normal donors, and this inhibition was additive with both FcεRI auto-inhibition and FcγRIIB-dependent inhibition.

Our study was focused on basophils. Basophils are few among the various effector cells that contribute to allergic manifestations. They may not be representative of other cells, especially mast cells that are dispersed throughout tissues where they differentiate into several types, depending on the environment. Allergic diseases can generate a variety of symptoms, depending on the cell types involved and on the location where the reaction occurs. Allergic diseases, however, may arise as a consequence of abnormalities that can affect specific regulatory mechanisms shared by several cell types. FcεRI-, FcγRIIB- and microbiota-dependent signaling that control the activation of basophils, but also that of other myeloid cells, are such mechanisms. If so, basophils may be used as readily accessible sample cells that can provide information on defects that possibly contribute to allergies.

Our study was focused on basophil activation. We found no differences between basophils from normal donors and basophils from allergic patients and, within the latter, between basophils from patients with different allergic diseases, when assessing basophil activation by measuring CD203c up-regulation in response to FcεRI engagement under several conditions. Our results do not imply that the secretory responses of basophils from patients of all five groups and normal donors were identical. Monitoring CD203c expression provides quantitative, by no qualitative information on basophil activation. Also, the five groups of patients were arbitrarily chosen, depending on symptomatology. Other criteria could have been chosen (*e.g.* the allergens involved), which might not have led to the same results. Finally, due to the small number of patients included in each group, one cannot exclude either that non-statistically significant differences observed here become significant when more patients are included.

Our study was focused on basophil activation *in vitro*. The aim of our work was to investigate whether the regulatory mechanisms known to operate in basophils from normal donors were functional in basophils from allergic patients. Such a study has limitations that are inherent to

any *in vitro* investigation. It also has advantages. The experimental conditions used permitted a comparison between basophils from unknown normal donors, and basophils from patients with different allergic diseases and allergic to a variety of allergens, which would not have been possible otherwise. Indeed, these far-from-physiology experimental conditions enabled all basophils to be challenged under the same conditions with the same reagents and therefore compared rigorously. Because they were obtained *in vitro*, our results neither exclude nor support the possibility that environmental influences to which basophils are exposed when circulating in the blood of patients might alter *in vivo* the regulatory mechanisms that we found to function normally *in vitro*. Such extrinsic factors were not investigated. Thus, our results exclude neither that too much specific IgE antibodies nor that not enough specific IgG antibodies were produced in response to allergens in some or all allergic patients.

Our study may contribute to sort out etiologic factors in allergy. Indeed, we found that no obvious intrinsic abnormality dampens regulatory mechanisms that control basophil activation in allergic patients. This was far from being expected. This finding does not exclude that these mechanisms may not be efficiently used, or even not used at all in allergic patients. It however excludes that intrinsic defects in basophils, whether resulting in a hyper-responsiveness to IgE-induced activation or in a hypo-responsiveness to inhibitory mechanisms, explain the allergic phenotype of the patients studied. As a consequence, the etiology of allergies may be searched for among extrinsic factors which could possibly impair one or several mechanisms that control the activation of basophils and possibly of other cells, in some or in all allergic diseases.

Our study may have therapeutic consequences. The use of probiotics capable of dampening the activation of effector cells of allergy (12) is supported by our observation that the inhibitory effect of *L. paracasei* is fully conserved in basophils from allergic patients. Noticeably, FcR-dependent and *L. paracasei*-induced inhibition do not compete with each other and, instead, have additive effects including in allergic patients, probably because they activate different inhibitory signaling pathways. This excludes that a prophylactic administration of probiotics to non-allergic individuals (17), might antagonize with physiological FcR-dependent regulation. Allergen immunotherapy, which was conceived as an allergen-based vaccine one century ago (36), elicits a robust allergen-specific IgG response rather than a tolerance state to allergen, and besides (or rather than) competing with IgE antibodies for allergen, IgG antibodies are likely to form allergen-antibody complexes that co-engage FcεRI and FcγRIIB on basophils (15, 16). Passively administered IgG antibodies or bispecific molecules capable of co-ligating, directly (18) or indirectly (19, 20) FcεRI with FcγRIIB have been generated, some of which with a modified Fc portion that has a markedly increased affinity for FcγRIIB (22). Our finding that FcεRI and FcγRIIB are fully functional on basophils from allergic patients strongly supports the development of such reagents. That no intrinsic abnormality dampens regulatory mechanisms that control basophil activation in allergic patients was indeed a requirement for therapeutic approaches based on antibody-induced regulatory mechanisms to be efficient. The spectacular success of novel anticancer immunotherapeutic tools collectively referred to as "checkpoint inhibitors" (37) has been possible because regulatory mechanisms that control anti-cancer immunity were not impaired in cancer patients. Our study therefore encourages "immune checkpoint modifiers" to be developed similarly against allergic and autoimmune diseases.

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**Table I. Experimental conditions used to assess basophil activation and inhibition of basophil activation**

Cells from each blood sample were submitted to the following 10 conditions:

**Overnight incubation with rIgE and:**

*PBS*

*L. paracasei (Lp)*

**Challenge with:**

RAHE F(ab') <sub>2</sub>	(1) 100% activation*	(2) Lp inhibition
RAHE IgG	(3) FcγRIIB inhibition	(4) Lp inhibition + FcγRIIB inhibition
MAR F(ab') <sub>2</sub>	(5) 100% activation**	(6) Lp inhibition
MAR IgG	(7) FcγRIIB inhibition	(8) Lp inhibition + FcγRIIB inhibition
MAR F(ab') <sub>2</sub> in excess	(9) FcεRI auto-inhibition	(10) Lp inhibition + FcεRI auto-inhibition

\* Induced via endogenous hIgE

\*\* Induced via exogenous rIgE

The table indicates the responses (activation or inhibition) of basophils previously shown to be induced by antibodies, antibody fragments or probiotic bacteria (10, 12). Responses induced by RAHE F(ab')<sub>2</sub> or MAR F(ab')<sub>2</sub> were used as 100% activation to calculate the percentage of inhibition induced by RAHE IgG, MAR IgG or *L. paracasei*.

## FIGURE LEGENDS

**Fig. 1:** Subjects and clinical parameters. (A) Numbers of individuals in the normal donors and the five patients groups. (B) Plasma IgE concentrations (yellow dots are A<sup>+</sup>-group patients with atopic dermatitis) and (C) basophil counts were for clinical purposes. Box-and-whisker plots show the median of the data, the lower and upper quartiles; Whiskers represent maximum 1.5 interquartile ranges (IQR).

**Fig. 2:** CD203c expression, receptor-bound human IgE and FcεRI expression in non-stimulated basophils from the 6 groups of subjects studied (A) not exposed to *L. paracasei*, or (B) incubated overnight with *L. paracasei*.

**Fig. 3:** FcγRIIA and FcγRII(A+B) expression in non-stimulated basophils from the 6 groups of subjects studied (A) not exposed to *L. paracasei*, or (B) incubated overnight with *L. paracasei*.

**Fig. 4:** Basophil activation induced by aggregating FcεRI-bound human IgE or rat IgE with corresponding F(ab')<sub>2</sub> fragments. The cartoons in the upper part of the figure schematize the experimental settings. They illustrate how Rabbit anti-human IgE F(ab')<sub>2</sub> fragments [RAHE F(ab')<sub>2</sub>] (A) and Mouse anti-Rat Immunoglobulin F(ab')<sub>2</sub> fragments [MAR F(ab')<sub>2</sub>] (B) can similarly aggregate basophil FcεRI when binding to human IgE and rat IgE, respectively. PBMCs from the 6 groups of subjects studied, passively sensitized with Rat IgE, were challenged with RAHE F(ab')<sub>2</sub> (A) or MAR F(ab')<sub>2</sub> (B). CD203c was monitored in basophils, and CD203c up-regulation observed following challenge was plotted as fold increases in the lower part of the figure. The dotted line (fold-increase = 1) corresponds to baseline levels (no challenge).

**Fig. 5:** Inhibition Rabbit anti-human IgE F(ab')<sub>2</sub> fragments-induced basophil activation by FcγRIIB, *L. paracasei* and FcγRIIB + *L. paracasei*. The cartoons in the upper part of the figure schematize the experimental settings used. They illustrate how Rabbit anti-human IgE F(ab')<sub>2</sub> fragments [RAHE F(ab')<sub>2</sub>] can aggregate basophil FcεRI when binding to human IgE, whereas intact Rabbit anti-human IgE antibodies (RAHE IgG) can coaggregate FcγRII and FcεRI on the same cells. PBMCs from the 6 groups of subjects studied were incubated with *L. paracasei* (B & C) or without (A). They were challenged with RAHE F(ab')<sub>2</sub> (B), or RAHE IgG (A & C). CD203c was monitored in basophils, and CD203c up-regulation induced by RAHE F(ab')<sub>2</sub> in cells not exposed to *L. paracasei* (not shown) was used as 100% response to calculate the % inhibition induced by RAHE IgG (A), *L. paracasei* (B), and RAHE IgG + *L. paracasei* (C) shown in the lower part of the figure.

**Fig. 6:** Inhibition of Mouse anti-Rat (MAR) F(ab')<sub>2</sub>-induced basophil activation by FcγRIIB, *L. paracasei* and FcγRIIB + *L. paracasei*, FcεRI, and FcεRI + *L. paracasei*. The cartoons in the upper part of the figure schematize the experimental settings used. They illustrate how Mouse anti-Rat Immunoglobulin F(ab')<sub>2</sub> fragments [MAR F(ab')<sub>2</sub>] can aggregate basophil FcεRI when binding to rat IgE, whereas intact Mouse anti-Rat Immunoglobulin antibodies (MAR IgG) can coaggregate FcγRII and FcεRI on the same cells. They also illustrate how an excess of MAR F(ab')<sub>2</sub> can over-aggregate FcεRI. PBMCs from the 6 groups of subjects studied were incubated with *L. paracasei* (B, C, F & G) or without (A, D & E). They were challenged with MAR F(ab')<sub>2</sub> (B), MAR IgG (A & C), a 9X excess of MAR F(ab')<sub>2</sub> (D & F) or a 18X excess of MAR F(ab')<sub>2</sub> (E & G). CD203c was monitored in basophils, and CD203c up-regulation induced by RAHE F(ab')<sub>2</sub> in cells not exposed to *L. paracasei* (not shown) was used as 100% response to calculate the % inhibition induced by MAR IgG (A), *L. paracasei* (B), MAR IgG + *L. paracasei* (C), MAR F(ab')<sub>2</sub> in excess (D & E), and MAR F(ab')<sub>2</sub> in excess + *L. paracasei* (F & G) shown in the lower part of the figure.

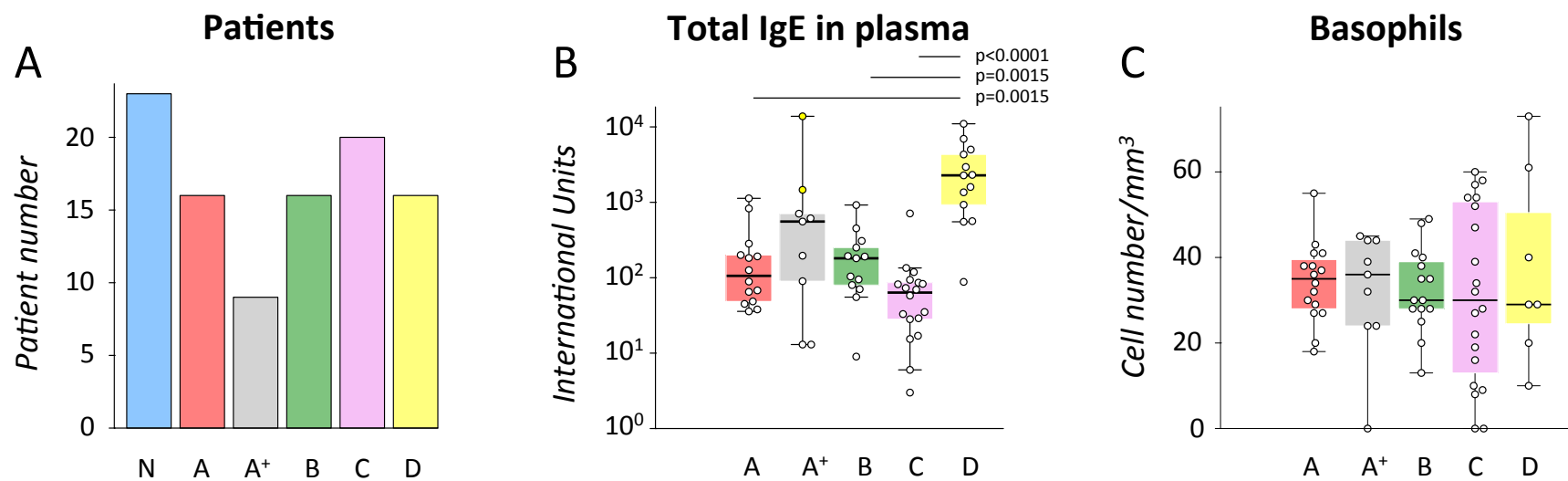


Fig. 1

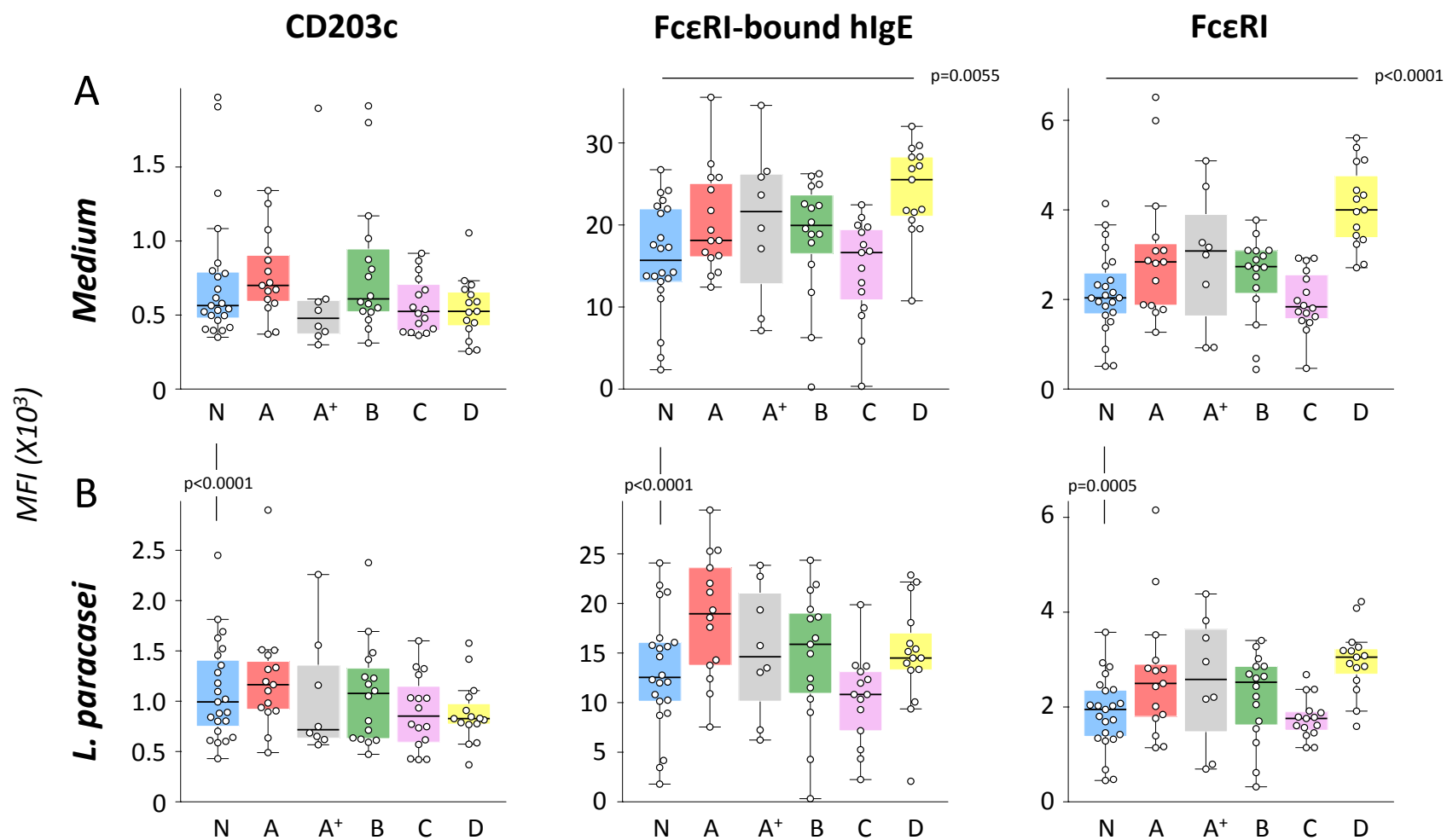


Fig. 2

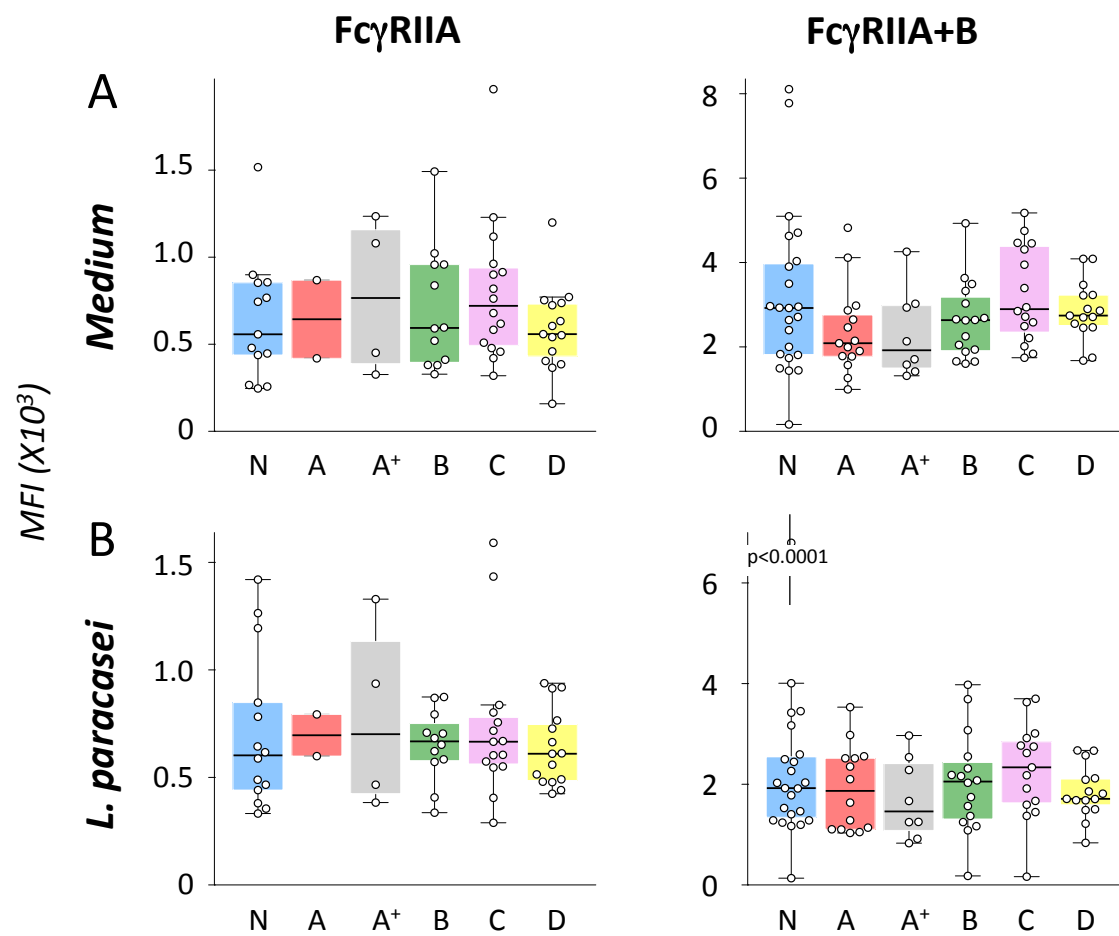


Fig. 3

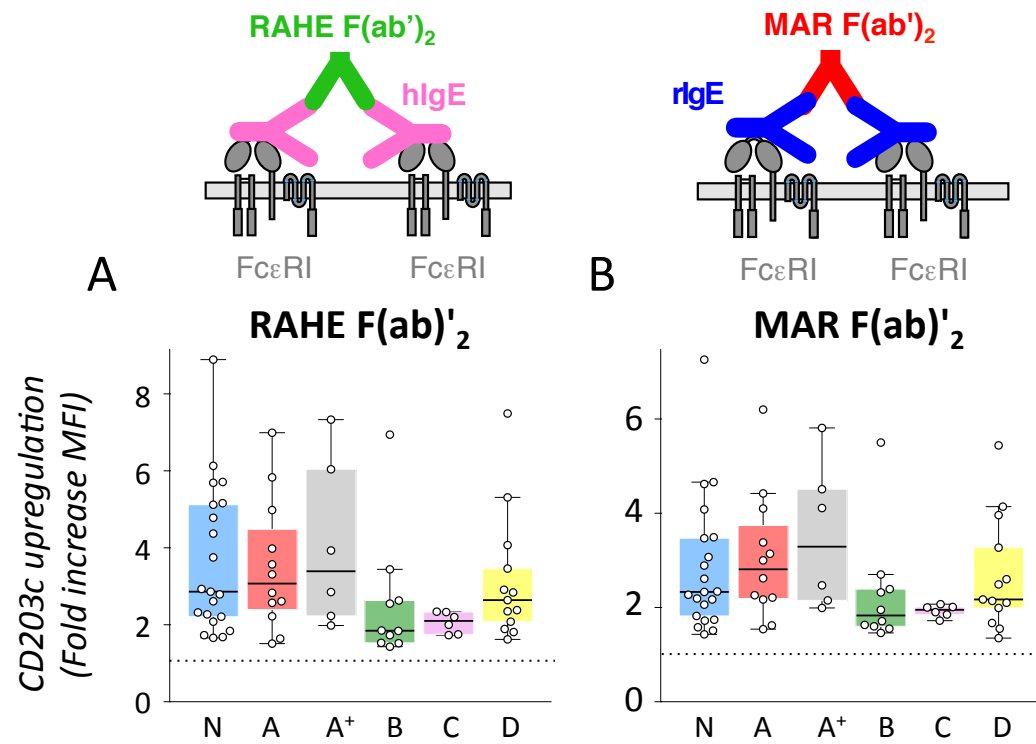
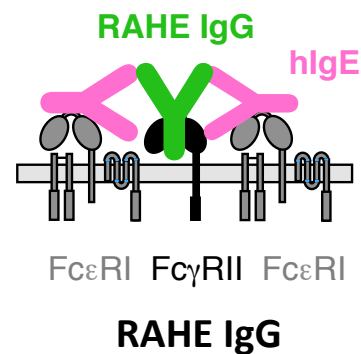
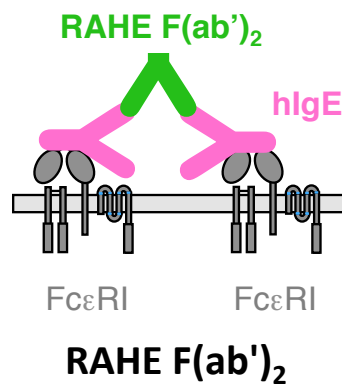


Fig. 4

% Inhibition RAHE F(ab')<sub>2</sub>-induced CD203c upregulation

Medium

*L. paracasei*



100%  
Response

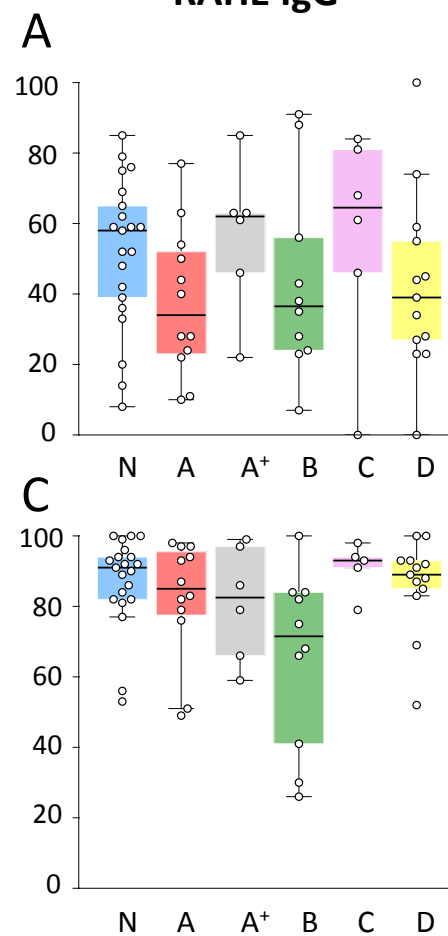
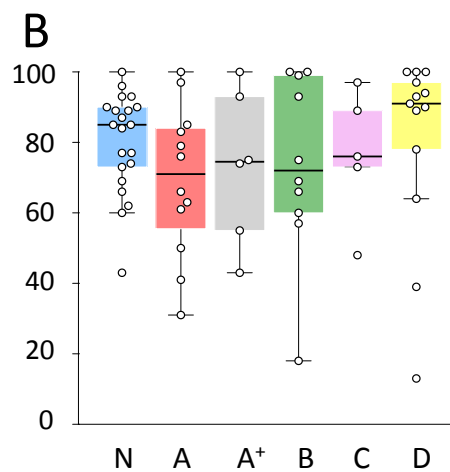


Fig. 5

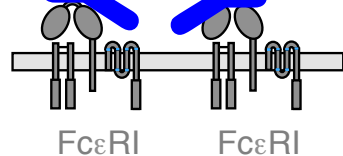
% Inhibition MAR F(ab')<sub>2</sub>-induced CD203c upregulation

Medium

*L. paracasei*

MAR F(ab')<sub>2</sub>

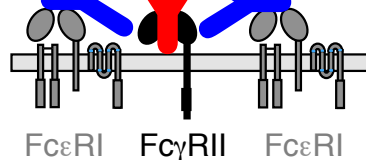
rgE



MAR F(ab')<sub>2</sub>

MAR IgG

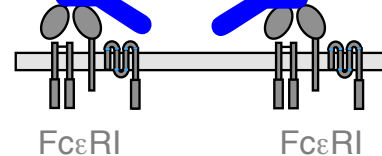
rgE



MAR IgG

MAR F(ab')<sub>2</sub>

rgE

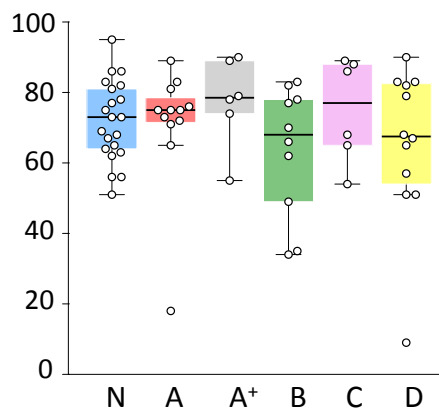


FcεRI

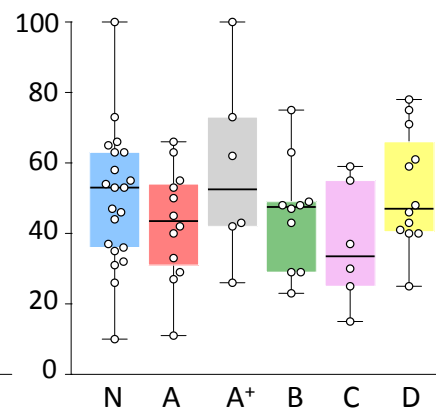
FcεRI

100%  
Response

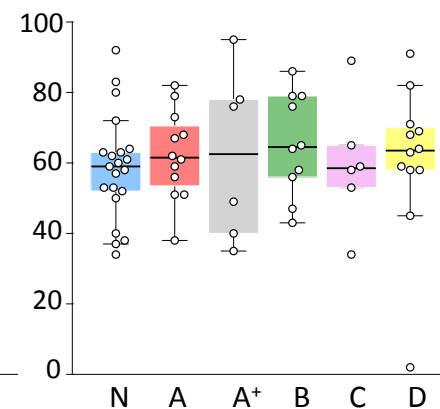
A



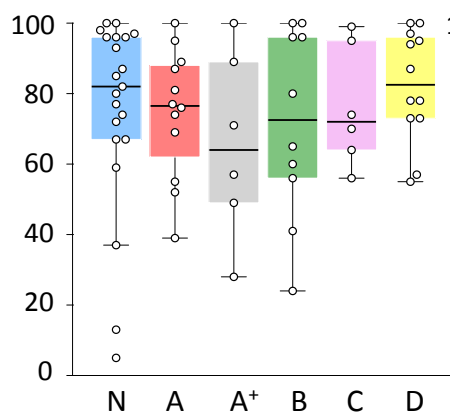
D



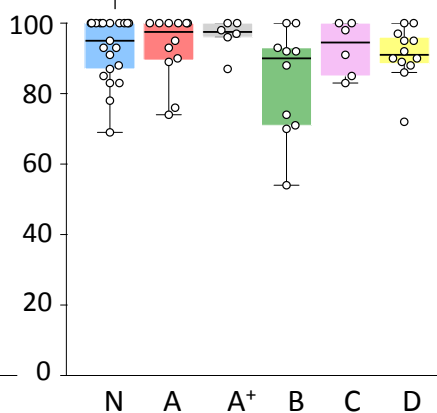
E



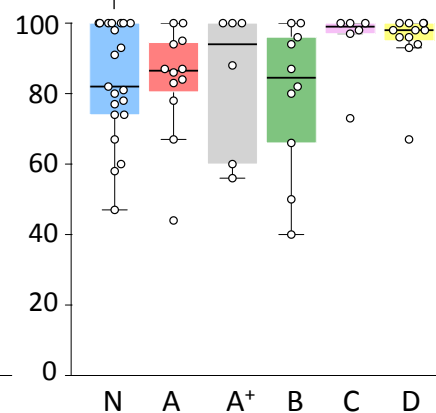
B



C



F



G

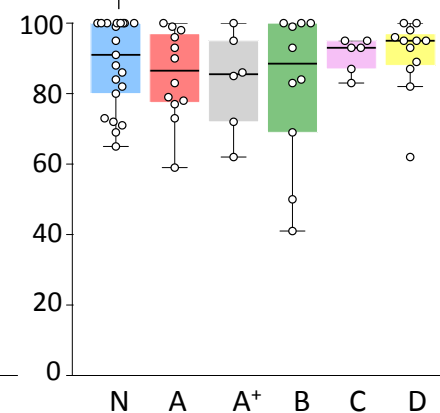


Fig. 6