

# Basophils from allergic patients are neither hyperresponsive to activation signals nor hyporesponsive to inhibition signals

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**Background:** Basophil activation contributes to inflammatory reactions, especially in allergy. It is controlled, both positively and negatively, by several mechanisms. High-affinity IgE receptors (FcεRI) generate a mixture of activation and inhibition signals when aggregated, the ratio of which depends on the concentration of allergen recognized by receptor-bound IgE. Low-affinity IgG receptors (FcγRIIA/B) generate inhibition signals when coengaged with FcεRI by allergen-antibody immune complexes. Commensal and probiotic bacteria, such as *Lactobacillus paracasei*, generate inhibition signals through still unclear mechanisms. **Objective:** We sought to investigate whether mechanisms that control, both positively and negatively, basophil activation,

which were unraveled and studied in basophils from healthy 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 or without *L. paracasei* and challenged under 10 experimental conditions. Basophils from healthy 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:** Patients' basophils expressed neither more FcεRI nor less FcγRIIB than basophils from healthy donors. They were neither hyperreactive to positive regulation nor hyporeactive to negative regulation, irrespective of the receptors or mechanisms involved and the allergic manifestations of the patients. **Conclusion:** Regulatory mechanisms that control basophil activation are fully functional in allergic patients. Intrinsic defects in these mechanisms do not explain allergic manifestations. Based on these mechanisms, immune checkpoint modifiers can be developed as novel therapeutic tools for allergy. (J Allergy Clin Immunol 2018;■■■■:■■■-■■■.)

**Key words:** Anaphylaxis, asthma, atopic dermatitis, basophil activation, chronic urticaria, FcεRI, FcγRIIB, lactobacilli, negative regulation, rhinitis

Basophils have been recognized to play critical roles in an increasing number of pathologic conditions. Particularly well known is their contribution to allergy as initiators of IgE-induced acute reactions. Basophils also account for late-phase<sup>1</sup> and chronic<sup>2</sup> 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.<sup>3,4</sup> Autoreactive IgE anti-double-stranded DNA antibodies associated with basophil activation were found in patients with systemic lupus erythematosus<sup>5</sup> and were correlated with the severity of nephritis.<sup>6</sup> Under normal conditions, however, basophils are under the control of a variety of regulatory mechanisms that might 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. However, FcεRI aggregation generates not only activation signals but also inhibition signals, the ratio of which depends on the degree of receptor aggregation (ie, 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

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Disclosure of potential conflict of interest: L. Cassard has received personal fees from Danone Research; has a patent (WO2009068997; "Use of a *L. casei* strain, for the preparation of a composition for inhibiting mast cell activation") issued; and has a patent ("Methods of inhibiting biological responses induced by a receptor that uses pi3k to signal in a cell") pending. A. Cotillard reports fees from Danone Research to her company for her consulting work. R. Bourdet-Sicard is an employee of Danone Research and has a patent (WO2009068997; "Use of a *L. casei* strain, for the preparation of a composition for inhibiting mast cell activation"). M. L. Albert is an employee of Genentech. M. Daëron has received a grant, personal fees, and nonfinancial support from Danone Research; has a patent (WO2009068997; "Use of a *L. casei* strain, for the preparation of a composition for inhibiting mast cell activation") issued; and has a patent ("Methods of inhibiting biological responses induced by a receptor that uses pi3k to signal in a cell") pending. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication January 26, 2017; revised November 1, 2017; accepted for publication November 21, 2017.

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0091-6749/\$36.00

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<https://doi.org/10.1016/j.jaci.2017.11.053>

**Abbreviations used**

hIgE: Human IgE  
MAR: Mouse anti-rat immunoglobulin antibody  
RAHE: Rabbit anti-human IgE antibody  
rIgE: Rat IgE  
SHIP1: SH2 domain-containing inositol 5-phosphatase 1

autoinhibition of FcεRI signaling depends on the SH2 domain-containing inositol 5 phosphatase 1 (SHIP1).<sup>7</sup> By hydrolyzing phosphatidylinositol 3,4,5-trisphosphate, which mediates the membrane recruitment of cytosolic signaling molecules with a pleckstrin homology domain,<sup>8</sup> SHIP1 inhibits distal signaling events, among which are Ca<sup>2+</sup> mobilization, activation of extracellular signal-regulated kinase 1/2, c-Jun N-terminal and p38 mitogen-activated protein kinases, and their biological consequences.

FcγRIIB-dependent negative regulation further controls mast cell<sup>9</sup> and basophil<sup>10</sup> activation. Because 1,000,000-fold more IgG than IgE (in milligrams per milliliter vs nanograms per milliliter) circulate in the bloodstream, allergens are likely to interact with FcεRI-bound IgE as IgG immune complexes rather than in isolation. Therefore allergens do not simply aggregate FcεRI; they also coaggregate FcεRI with low-affinity IgG receptors expressed on the same cell. These include activating (FcγRIIA) and inhibitory (FcγRIIB) receptors. Because 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 coengaged by immune complexes on basophils from healthy donors.<sup>10</sup> 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, which, when phosphorylated, has high affinity for the SH2 domain of SHIP1.<sup>11</sup>

Increasingly, commensal bacteria have 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 PBMCs are exposed to specific strains of *Lactobacillus paracasei* before challenge.<sup>12</sup> Inhibition requires direct contact between cells and bacteria, but the molecular mechanism accounting for inhibition was not elucidated.

These regulatory mechanisms have been regarded increasingly as potential therapeutic tools for controlling the effector phase of inflammation, especially in allergic patients. Allergen immunotherapy is currently thought to depend on the generation of regulatory T cells that promote a state of “tolerance” to allergen by dampening T<sub>H</sub>2 responses.<sup>13</sup> Nevertheless, it induces high amounts of allergen-specific IgG antibodies that have long been believed to compete with specific IgE for antigen.<sup>14</sup> IgG antibodies found in the sera of patients treated by immunotherapy were shown recently to dampen basophil activation through a FcγRIIB-dependent mechanism.<sup>15,16</sup> Consumption of probiotic bacteria has been proposed as a prophylactic measure to prevent either onset of allergies in healthy subjects or the outcome of allergic symptoms in allergic patients.<sup>17</sup> Bispecific molecules capable of coligating FcγRIIB with either FcεRI<sup>18</sup> or FcεRI-bound IgE<sup>19,20</sup> have been generated as potential new treatments of allergies, and genetically engineered IgG antibodies

have been produced, among which are anti-IgE antibodies, the affinity of which for FcγRIIB is several hundred-fold higher than that for native antibodies.<sup>21,22</sup>

The efficacy of these therapeutic approaches implies that the regulatory mechanisms on which they lie are functional in allergic patients. These mechanisms were indeed described and studied in basophils from healthy donors but they were not examined in allergic patients. If, for whatever reason, 1 or several of these mechanisms are deficient in patients with 1 or several allergic conditions, therapeutic tools can be disqualified for 1 or several allergic diseases. On the other hand, finding that negative regulation is impaired in some or all allergic patients, irrespective of the mechanism, can provide a clue to understanding the cause of the allergies. What makes patients allergic and/or what prevents nonallergic subjects from being allergic is not known, and one possibility is that negative regulation is operational and protective in nonallergic subjects but impaired in allergic patients.

Therefore we 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, irrespective of their pathologic conditions.

## METHODS

### Patients and healthy donors

A total of 77 patients (57 female and 20 male patients; 18–77 years old [mean, 44 years]) were enrolled in the study over a 3-month period (from October 22, 2013, to January 29, 2014). Inclusion criteria were as follows: age greater than 18 years, presence of an allergic disease, prescription of a blood test, and agreement to be enrolled in the study by giving their informed consent. Exclusion criteria were as follows: inability to provide consent, presence of evolutive neoplasia or infectious or inflammatory disease, receipt of glucocorticoid or antihistamine medication less than 1 week earlier, and receipt of immunosuppressive treatment, irrespective of 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 on clinical symptoms, skin test results, and biological measurements. Patients were classified by clinicians into 5 groups as a function of diagnosis (Fig 1, A):

- **Group A (16 patients), respiratory allergy:** patients sensitized to aeroallergens and/or with a personal history of atopy with recurrent spasmodic coryza and/or asthma with bronchial hyperreactivity defined by a 20% FEV<sub>1</sub> increase after inhalation of 200 µg of salbutamol, as specified by Bousquet et al<sup>23</sup> and Bateman et al<sup>24</sup>;
- **Group B (16 patients), anaphylaxis antecedents:** patients with generalized urticaria, respiratory obstruction, severe tachycardia, blood pressure decrease and/or loss of consciousness occurring immediately after Hymenoptera sting, food ingestion or drug intake corresponding to grades 2 to 3, as specified by Sampson et al<sup>25</sup>;
- **Group C (20 patients), chronic urticaria:** patients with chronic urticaria symptoms, as specified by Zuberbier et al,<sup>26</sup> for more than 6 weeks;
- **Group D (16 patients), atopic dermatitis:** patients with long-lasting dermatitis of childhood onset and persistence into adulthood affecting the head and neck and, in some patients, the popliteal and antecubital fossae, as specified by Eichenfield et al<sup>27</sup>;
- **Group A<sup>+</sup> (9 patients), respiratory allergy plus one other allergic condition:** 1 case of anaphylaxis, 2 cases of chronic urticaria, 5 cases of food allergies, and 1 case of Quincke edema; and
- **Group N:** 23 anonymous healthy blood donors from the Etablissement Français du Sang who also provided written informed consent for their blood to be included in the research program.

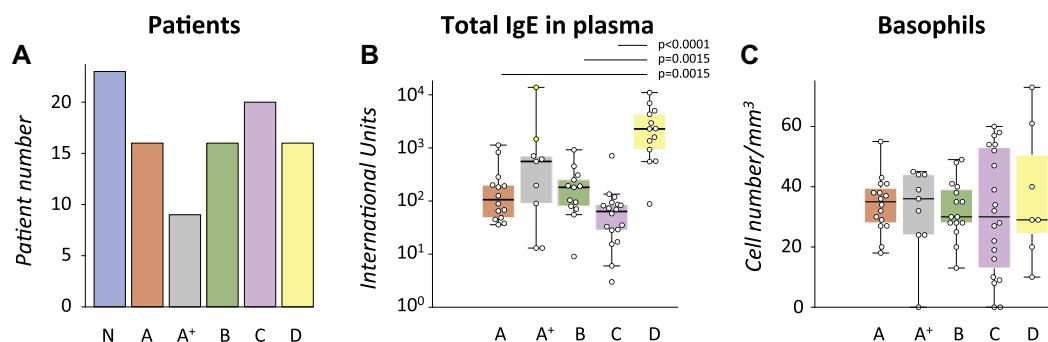


**FIG 1.** Subjects and clinical parameters. **A**, Numbers of subjects in the healthy donor and 5 patient groups. **B** and **C**, Plasma IgE concentrations (yellow dots are Group A<sup>+</sup> patients with atopic dermatitis) and basophil counts were used for clinical purposes. Box-and-whisker plots show medians and lower and upper quartiles of the data; whiskers represent maximum 1.5 interquartile ranges.

In practice, one 5- to 10-mL blood sample was collected in EDTA during a blood test that had been prescribed to investigate the clinical condition of the patient independent of the study. One blood sample only from each patient was examined. Blood from 1 to 5 patients was tested every day together with 1 blood sample drawn on the same day from a healthy 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 insufficient basophils for being analyzed and (2) 2 Group N, 3 Group A, 2 Group A<sup>+</sup>, 6 Group B, 10 Group C, and 3 Group D samples in which basophils were not activated on FcεRI aggregation by 1 or both anti-IgE reagents used. Data from 2 donors were also missing because of a technical error. Noticeably, for the above reasons, only 6 of 20 Group C samples could be exploited. Blood samples were numbered in chronologic order. Experiments were performed blindly because only clinical doctors knew the diagnosis. No diagnostic information was disclosed until experiments with blood samples from all subjects were completed and all data were filed and analyzed. Conversely, experimental results were not disclosed to clinicians until the investigation was complete.

## Experimental procedures

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

**Bacteria.** *L. paracasei* was the same strain (CNCM I-1518) used in our previous work.<sup>12</sup> It was cultured in Mann-Rogosa-Sharpe broth at 37°C for 48 hours, washed, resuspended in PBS, and adjusted to an OD<sub>600</sub> value of 2. Ten microliters of *L. paracasei* was added to 90 μL of PBMCs together with rIgE. Therefore cells were incubated overnight with bacteria.

**Challenge.** Cells were washed extensively and stimulated for 20 minutes at 37°C with equimolar concentrations of the following 4 reagents: (1) 15 μg/mL rabbit anti-human IgE antibody (RAHE IgG; Dako Cytomation, Trappes, France); (2) 10 μg/mL F(ab')<sub>2</sub> fragments obtained by means of peptic digestion of the same antibodies (RAHE F(ab')<sub>2</sub>); (3) 15 μg/mL mouse anti-rat immunoglobulin antibody (MAR IgG; Jackson ImmunoResearch, West Grove, Pa); and (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 previously described,<sup>28</sup> and basophil activation was monitored by using CD203c upregulation, as previously described.<sup>10</sup>

**Immunofluorescence analysis.** Cells were stained with phycoerythrin-conjugated anti-CD203c antibodies (Immunotech, Marseille, France), allophycocyanin-conjugated anti-FcεRIα antibodies (eBioscience, San Diego, Calif), fluorescein isothiocyanate-conjugated anti-human IgE (hIgE) antibodies (Sigma-Aldrich, St Louis, Mo), fluorescein isothiocyanate-conjugated anti-FcγRIIA (IV.3; STEMCELL Technologies, Vancouver, British Columbia, Canada), or Vioblue-anti-FcγRIIA+B (2E1) antibodies (Miltenyi Biotech, 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 TO-PRO-3 (Life Technologies, Grand Island, NY). Fluorescence was assessed by using flow cytometry.

## Statistical analysis

Statistical analyses were performed with SAS 9.3 (SAS Institute, Cary, NC) and R 3.3.0 software. Either raw values or variations between 2 conditions were analyzed. Wilcoxon signed-rank tests were used to compare 2 conditions in healthy 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* values of less than .05 were considered significant.

## RESULTS

FcεRI and FcγRIIA/B expression, anti-IgE-induced activation, FcεRI autoinhibition, and FcγRIIB-dependent inhibition were compared in basophils from healthy blood donors (Group N) and basophils from patients with respiratory allergy (Group A), anaphylaxis antecedents (Group B), chronic urticaria (Group C), atopic dermatitis (Group D), or respiratory allergy plus 1 other allergic condition (Group A<sup>+</sup>). The effects of *L. paracasei* were examined on FcεRI and FcγRIIA/B expression, anti-IgE-induced activation, FcεRI autoinhibition, and FcγRIIB-dependent inhibition in basophils from the same 6 groups of donors.

## Basal parameters

As expected<sup>29</sup> and as observed previously,<sup>28</sup> plasma IgE levels were significantly greater in patients with atopic dermatitis (Group D) than in other allergic patients (Fig 1, B). Consequently, basophils from Group D patients displayed more FcεRI-bound hIgE and expressed more FcεRI than basophils from healthy donors (Fig 2, A). No statistically significant differences in basophil numbers were observed among the 5 groups of patients

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

|                                   | Overnight incubation with rIgE and |                                                            |
|-----------------------------------|------------------------------------|------------------------------------------------------------|
|                                   | PBS                                | <i>L. paracasei</i>                                        |
| Challenge with:                   |                                    |                                                            |
| RAHE F(ab') <sub>2</sub>          | (1) 100% activation*               | (2) <i>L. paracasei</i> inhibition                         |
| RAHE IgG                          | (3) FcγRIIB inhibition             | (4) <i>L. paracasei</i> inhibition + FcγRIIB inhibition    |
| MAR F(ab') <sub>2</sub>           | (5) 100% activation†               | (6) <i>L. paracasei</i> inhibition                         |
| MAR IgG                           | (7) FcγRIIB inhibition             | (8) <i>L. paracasei</i> inhibition + FcγRIIB inhibition    |
| MAR F(ab') <sub>2</sub> in excess | (9) FcεRI autoinhibition           | (10) <i>L. paracasei</i> inhibition + FcεRI autoinhibition |

Cells from each blood sample were submitted to the above 10 conditions (numbered in parentheses). The table indicates responses (activation or inhibition) of basophils previously shown to be induced by antibodies, antibody fragments, or probiotic bacteria.<sup>10,12</sup> 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*.

\*Induced through endogenous hIgE.

†Induced through exogenous rIgE.

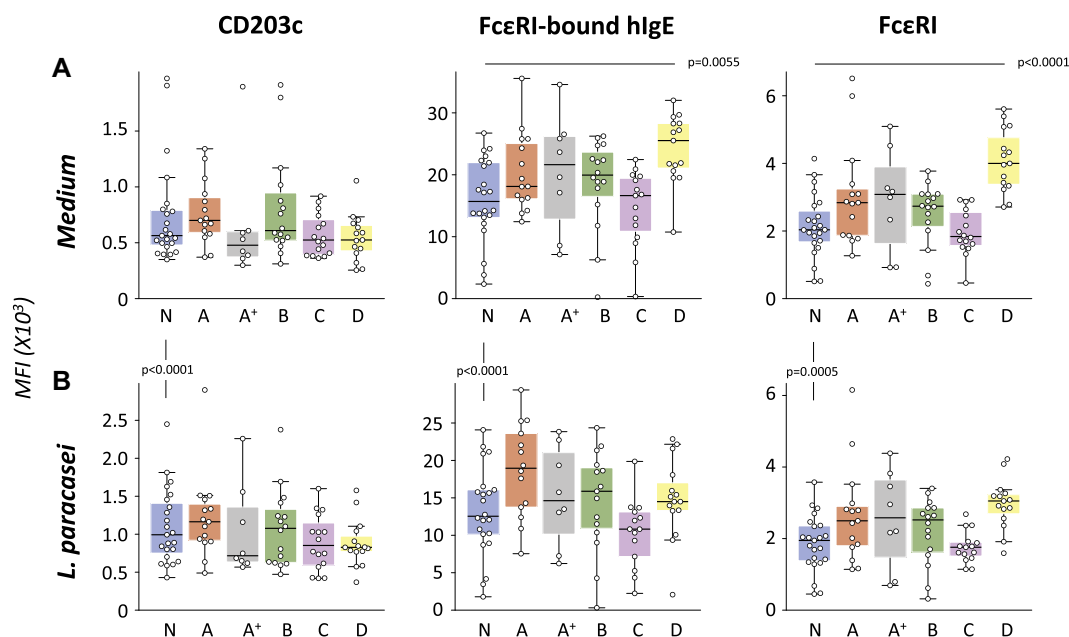


**FIG 2.** CD203c expression, receptor-bound hIgE, and FcεRI expression in nonstimulated basophils from the 6 groups of subjects studied which were not exposed to *L. paracasei* (A) or incubated overnight with *L. paracasei* (B). MFI, Mean fluorescence intensity.

(Fig 1, C). No statistically significant difference in CD203c expression was observed between basophils from the 5 groups of patients and those from healthy donors (Fig 2, A).

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

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

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

*L. paracasei* similarly affected basophils from healthy donors and basophils from allergic patients. However, *L. paracasei*-induced

differences that were statistically different in basophils from healthy donors were not statistically different in basophils from allergic patients (Fig 2, B, and 3, B). Also, both increased FcεRI expression and increased FcεRI-bound IgE levels seen in basophils from patients with atopic dermatitis decreased to levels that were not statistically different from levels seen in basophils from healthy donors and other patients after exposure to *L. paracasei* (Fig 2, B).

### 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 upregulation of CD203c with flow cytometry (Table I, condition 1). Indeed, RAHE F(ab')<sub>2</sub> increased CD203c expression 3-fold in basophils from healthy donors. A similar 2- to 3-fold upregulation of CD203c expression was induced under the same conditions in basophils from the 5 groups of patients (Fig 4, A). However, one should remember 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 to basophils from healthy

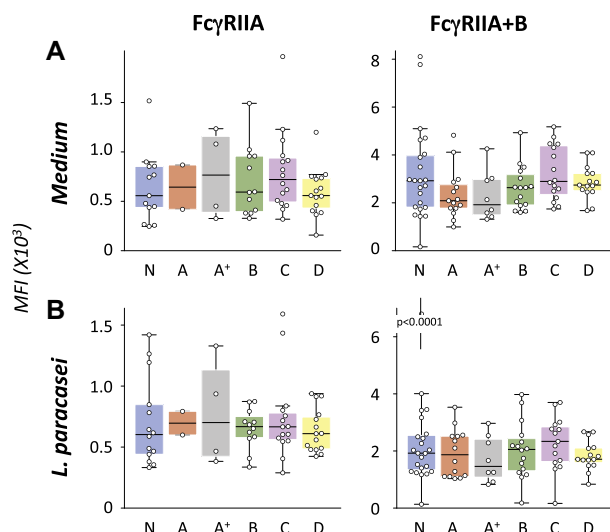


**FIG 3.** FcγRIIA and FcγRII(A+B) expression in nonstimulated basophils from the 6 groups of subjects studied which were not exposed to *L paracasei* (A) or incubated overnight with *L paracasei* (B). MFI, Mean fluorescence intensity.

donors, and the slight differences in CD203c upregulation seen in basophils from the various groups of patients were not statistically significant.

All FcεRI are not occupied by endogenous hIgE. Therefore cells were sensitized passively with rIgE. Human FcεRI can bind rIgE with a similarly high affinity as hIgE.<sup>30</sup> 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.<sup>10</sup> MAR F(ab')<sub>2</sub> increased CD203c expression 2- to 3-fold in basophils from healthy donors, as well as in basophils from the 5 groups of patients (Fig 4, B). Basophils from the Group C patients that were not activated by RAHE F(ab')<sub>2</sub> were not activated by MAR F(ab')<sub>2</sub> either. Differences in CD203c upregulation seen in basophils from the various groups of patients were not statistically significant.

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

When recognizing receptor-bound hIgE, intact RAHE IgG antibodies coaggregate FcεRI through their Fab portions and FcγRIIA+B through their Fc portion on the same cell (Table I, condition 2). Under this condition, FcγRIIB inhibit FcεRI-dependent activation.<sup>10</sup> Inhibition can be monitored by comparing CD203c upregulation induced under condition 2 with CD203c upregulation induced under condition 1. RAHE IgG induced a 40% to 60% inhibition in healthy donors. Inhibitions of comparable magnitudes were observed in basophils from the 5 groups of patients (Fig 5, A). Differences between healthy donors and patients of the 5 groups were not statistically significant.

When recognizing receptor-bound rIgE, intact MAR IgG antibodies coaggregate FcεRI through their Fab portions and FcγRIIB+A through their Fc portion on the same cell (Table I, condition 7), and FcγRIIB inhibit FcεRI-dependent activation. Inhibition was monitored by comparing CD203c upregulation induced under condition 7 with CD203c upregulation induced

under condition 5. MAR IgG induced a ±70% inhibition in basophils from healthy donors and inhibitions of comparable magnitudes in basophils from the 5 groups of patients (Fig 6, A).

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

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

### MAR F(ab')<sub>2</sub>-induced FcεRI autoinhibition

As expected, CD203c upregulation displayed a bell-shaped curve when basophils passively sensitized with rIgE were challenged with increasing concentrations of MAR F(ab')<sub>2</sub> (data not shown). Indeed, an excess of ligand induces dose-dependent inhibition of cell activation that depends on the lipid phosphatase SHIP1.<sup>7</sup> This FcεRI autoinhibition was assessed by comparing CD203c upregulation induced by an optimal concentration of 10 μg/mL (1X) MAR F(ab')<sub>2</sub> (Table I, condition 1) and CD203c upregulation induced by 2 supraoptimal concentrations (9X and 18X) of MAR F(ab')<sub>2</sub> (Table I, condition 9). 9X MAR F(ab')<sub>2</sub> induced a ±50% inhibition in basophils from healthy donors (Fig 6, D), and 18X MAR F(ab')<sub>2</sub> induced an inhibition of ±60% (Fig 6, E). FcεRI autoinhibition was of comparable magnitudes in basophils from healthy donors and basophils from patients. One notices that the nonstatistically 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>.

### FcγRIIB-dependent inhibition or FcεRI autoinhibition plus *L paracasei*-induced inhibition

Unlike the well-established SHIP1-mediated mechanism of FcεRI autoinhibition and FcγRIIB-dependent inhibition, the molecular mechanism by which *L paracasei* inhibits basophil activation is poorly known. We wondered whether *L paracasei*-induced inhibition would compete with SHIP1-mediated inhibition. PBMCs that had been exposed to *L paracasei* were challenged with RAHE IgG (Table I, condition 4), MAR IgG (Table I, condition 8), or supraoptimal concentrations of MAR F(ab')<sub>2</sub> (Table I, condition 10). Inhibition of CD203c upregulation observed under these conditions was compared with inhibition induced by the same ligands in cells not exposed to *L paracasei* (Table I, conditions 3, 7, and 9, respectively) and with inhibition induced by *L paracasei* alone (Table I, conditions 2 and 6).

*L paracasei* plus RAHE IgG-induced inhibition of normal basophil activation (Fig 5, C) was much greater than inhibition induced by RAHE IgG alone (Fig 5, A) and slightly

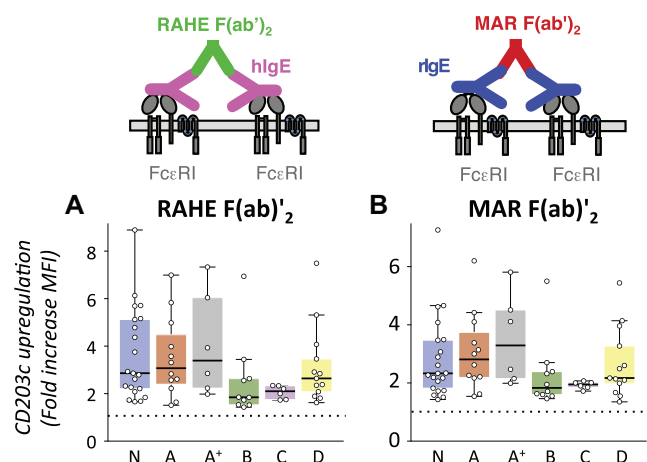


**FIG 4.** Basophil activation induced by aggregating FcεRI-bound hIgE or rIgE with corresponding F(ab')<sub>2</sub> fragments. *Cartoons in the upper part of the figure* schematize the experimental settings. They illustrate how RAHE F(ab')<sub>2</sub> (**A**) and MAR F(ab')<sub>2</sub> (**B**) can similarly aggregate basophil FcεRI when binding to hIgE and rIgE, respectively. PBMCs from the 6 groups of subjects studied and passively sensitized with rIgE were challenged with RAHE F(ab')<sub>2</sub> (Fig 4, **A**) or MAR F(ab')<sub>2</sub> (Fig 4, **B**). CD203c was monitored in basophils, and CD203c upregulation observed after challenge was plotted as the fold increase in the lower part of the figure. The dotted line (fold increase = 1) corresponds to baseline levels (no challenge). MFI, Mean fluorescence intensity.

greater than inhibition induced by *L paracasei* alone (Fig 5, **B**). *L paracasei* + RAHE IgG–induced inhibitions were similarly greater than inhibition induced by *L paracasei* or RAHE IgG alone in basophils from patients and in basophils from healthy donors, except in Groups B and D, where inhibition induced by *L paracasei* plus RAHE IgG (Fig 5, **C**) was similar to inhibition induced by *L paracasei* alone (Fig 5, **B**). However, differences between healthy donors and the 5 groups of patients were not statistically significant.

*L paracasei* plus MAR IgG–induced inhibition of normal basophil activation (Fig 6, **C**) was also much greater than inhibition induced by MAR IgG alone (Fig 6, **A**) or *L paracasei* alone (Fig 6, **B**). *L paracasei* plus MAR IgG–induced inhibition was similarly greater than inhibition induced by *L paracasei* or MAR IgG alone in basophils from patients and basophils from healthy donors.

Inhibition of normal basophil activation induced by *L paracasei* and a supraoptimal concentration of MAR F(ab')<sub>2</sub> (Fig 6, **F** and **G**) was greater than inhibition induced by an excess of MAR F(ab')<sub>2</sub> alone (Fig 6, **D** and **E**) but of the same magnitude as inhibition induced by *L paracasei* alone (Fig 6, **B**). 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, either positively or negatively, activation of basophils from healthy donors. The unexpected overall result is that, when compared with basophils from healthy donors, patients' basophils were neither hyperreactive to positive regulation nor hyporeactive to negative regulation, irrespective of their allergic manifestations. This

result has important consequences for our understanding of the cause of allergies and for new therapeutic strategies being developed.

First, we noticed both expected and unexpected abnormalities in 2 groups of patients. As expected, basophils from patients with atopic dermatitis (Group D) expressed more FcεRI and displayed more FcεRI-bound hIgE than basophils from healthy donors or other patients. These differences were correlated with increased plasma IgE levels, which are associated classically with atopic dermatitis.<sup>29</sup> It is well known that FcεRI expression is upregulated by IgE,<sup>31</sup> which slows the intracellular degradation of receptors occupied by IgE.<sup>32</sup>

Unexpected basophil abnormalities were observed in some but not all patients with chronic urticaria (Group C). Three among the 19 patients included in this group (1 of the 20 Group C samples could not be processed for irrelevant technical reasons) had basophil numbers too low for study, and the basophils of 10 others did not respond to FcεRI aggregation, either 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 healthy donor basophils to FcεRI aggregation. Therefore Group C might contain 2 subgroups of patients, 68% (13/19) of whom have low basophil numbers or hyporeactive basophils, and 32% (6/19) of whom have 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 found in the sera of 30% to 60% of patients with chronic urticaria,<sup>33,34</sup> which are thought to trigger basophil and/or mast cell activation responsible for clinical manifestations. Whether such autoantibodies were present in the sera of patients with abnormal basophil counts or functions is not known. Both low basophil numbers or hyporeactive basophils could result from a chronic stimulation by anti-FcεRI autoantibodies. In addition, an unknown factor present in the serum of patients with active chronic idiopathic urticaria was found recently, which inhibits FcεRI-dependent histamine release by basophils.<sup>35</sup>

Remarkably, FcεRI expression and functions were unaltered in basophils from all other patients. Basophils from all patients except those with atopic dermatitis expressed comparable amounts of FcεRI as basophils from healthy donors. Basophils from all patients except half of the patients with chronic urticaria but including patients with atopic dermatitis with increased FcεRI expression were activated similarly as basophils from healthy donors by an optimal concentration of F(ab')<sub>2</sub> anti-IgE, irrespective of whether through endogenous hIgE or exogenous rIgE. Activation of basophils from all patients, including hyporesponsive basophils from half of the patients with chronic urticaria, was inhibited similarly by an excess of MAR F(ab')<sub>2</sub> fragments as basophils from healthy donors. Therefore basophils from allergic patients did not overexpress FcεRI, were not overresponsive to FcεRI aggregation–triggered activation signals, and were not hyporesponsive to FcεRI hyperaggregation–triggered inhibition signals.

Likewise, FcγRIIA and FcγRIIB expression and function were unaltered in basophils from all patients. FcγRIIA expression was comparable on basophils from patients as on basophils from healthy donors. Whether the activating properties of this immunoreceptor tyrosine-based activation motif–containing receptor were as modest as previously observed in basophils from healthy donors<sup>10</sup> was not examined in basophils from

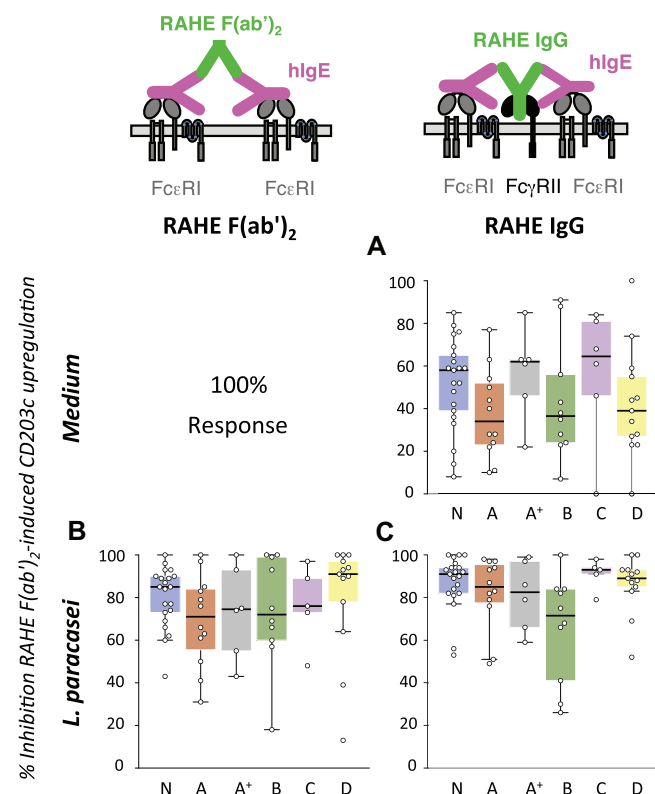


**FIG 5.** Inhibition of rabbit anti-human IgE F(ab')<sub>2</sub> fragment-induced basophil activation by FcγRIIB, *L. paracasei*, and FcγRIIB plus *L. paracasei*. Cartoons in the upper part of the figure schematize the experimental settings used. They illustrate how RAHE F(ab')<sub>2</sub> can aggregate basophil FcεRI when binding to hIgE, whereas intact RAHE IgG can coaggregate FcγRII and FcεRI on the same cells. PBMCs from the 6 groups of subjects studied were incubated with (B and C) or without (A) *L. paracasei*. They were challenged with RAHE F(ab')<sub>2</sub> (Fig 5, B) or RAHE IgG (Fig 5, A and C). CD203c was monitored in basophils, and CD203c upregulation induced by RAHE F(ab')<sub>2</sub> in cells not exposed to *L. paracasei* (data not shown) was used as a 100% response to calculate the percentage inhibition induced by RAHE IgG (Fig 5, A), *L. paracasei* (Fig 5, B), and RAHE IgG plus *L. paracasei* (Fig 5, C).

patients. FcγRIIA+B expression (ie, because of the markedly unbalanced expression of the 2 receptors, primarily FcγRIIB expression) was comparable on basophils from patients and those from healthy donors. Whether assessed with RAHE IgG or MAR IgG, FcγRIIB-dependent negative regulation of FcεRI-dependent activation was similar in basophils from patients of all 5 groups as in those from healthy donors. Therefore basophils from allergic patients 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* had a similar affect on basophils from all groups of patients and those from healthy donors. Basal CD203c expression was increased similarly, whereas FcεRI-bound hIgE and FcγRIIB expression was similarly decreased; FcεRI and FcγRIIA expression was similarly not changed. Noticeably, *L. paracasei* decreased both augmented FcεRI expression and FcεRI-bound hIgE levels back to normal in basophils from patients with atopic dermatitis. *L. paracasei* inhibited similarly FcεRI-dependent activation in basophils from all patients and healthy donors, irrespective of 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

autoinhibition induced by an excess of ligand not only in basophils from healthy 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 healthy donors and those from patients, despite the decreased FcγRIIB expression induced by *L. paracasei*. Therefore *L. paracasei* inhibited equally efficiently FcεRI-dependent activation in basophils from allergic patients and those from healthy donors, and this inhibition was additive with both FcεRI autoinhibition and FcγRIIB-dependent inhibition.

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

Our study was focused on basophil activation. We found no differences between basophils from healthy donors and those from allergic patients and, among the latter, between basophils from patients with different allergic diseases when assessing basophil activation by measuring CD203c upregulation in response to FcεRI engagement under several conditions. Our results do not imply that the secretory responses of basophils from patients of all 5 groups and healthy donors were identical. Monitoring CD203c expression provides quantitative but no qualitative information on basophil activation. Also, the 5 groups of patients were chosen arbitrarily, depending on symptomatology. Other criteria could have been chosen (eg, the allergens involved), which might not have led to the same results. Finally, because of the small number of patients included in each group, one cannot exclude that nonstatistically significant differences observed here might 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 healthy donors were functional in basophils from allergic patients. Such a study has limitations inherent to any *in vitro* investigation, but it also has advantages. The experimental conditions used permitted a comparison between basophils from unknown healthy donors and basophils from patients with different allergic diseases and allergic responses to a variety of allergens, which would not have been possible otherwise. Indeed, these experimental conditions, which were far from physiologic 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.

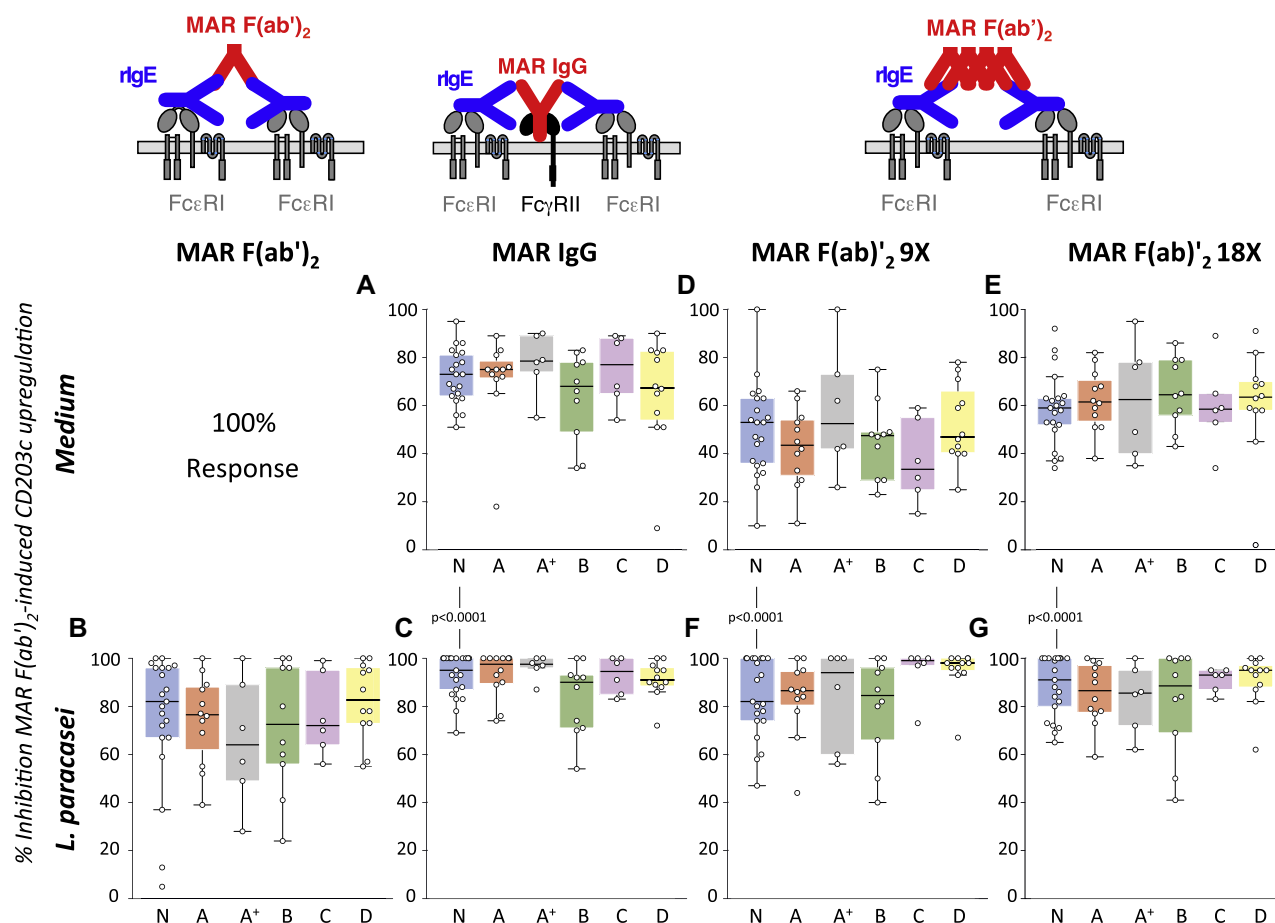


**FIG 6.** Inhibition of mouse anti-Rat F(ab')<sub>2</sub>-induced basophil activation by FcγRIIB, *L paracasei*, and FcγRIIB plus *L paracasei*, FcεRI, and FcεRI plus *L paracasei*. Cartoons in the upper part of the figure schematize the experimental settings used. They illustrate how MAR F(ab')<sub>2</sub> fragments can aggregate basophil FcεRI when binding to rIgE, whereas intact 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 overaggregate FcεRI. PBMCs from the 6 groups of subjects studied were incubated with (B, C, F, and G) or without (A, D, and E) *L paracasei*. They were challenged with MAR F(ab')<sub>2</sub> (Fig 6, B), MAR IgG (Fig 6, A and C), a 9× excess of MAR F(ab')<sub>2</sub> (Fig 6, D and F), or a 18× excess of MAR F(ab')<sub>2</sub> (Fig 6, E and G). CD203c was monitored in basophils, and CD203c upregulation induced by RAHE F(ab')<sub>2</sub> in cells not exposed to *L paracasei* (data not shown) was used as a 100% response to calculate the percentage inhibition induced by MAR IgG (Fig 6, A), *L paracasei* (Fig 6, B), MAR IgG plus *L paracasei* (Fig 6, C), MAR F(ab')<sub>2</sub> in excess (Fig 6, D and E), and MAR F(ab')<sub>2</sub> in excess plus *L paracasei* (Fig 6, F and G).

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 might contribute to sorting out causative 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 might not be efficiently used or even not used at all in allergic patients. However, it excludes that intrinsic defects in basophils, irrespective of whether resulting in hyperresponsiveness to IgE-induced activation or hyporesponsiveness to inhibitory mechanisms, explain the allergic phenotype of the patients studied. As a consequence, the cause of allergies can be searched for among extrinsic factors that could impair 1 or several mechanisms that control the activation of basophils and possibly of other cells in some or all allergic diseases.

Our study can have therapeutic consequences. The use of probiotics capable of dampening the activation of effector cells of allergy<sup>12</sup> 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 nonallergic subjects<sup>17</sup> might antagonize physiologic FcR-dependent regulation. Allergen immunotherapy, which was conceived of as an allergen-based vaccine 1 century ago,<sup>36</sup> elicits a robust allergen-specific IgG response rather than a tolerance state to allergen, and in addition to (or rather than) competing with IgE antibodies for allergen, IgG antibodies are likely to form allergen-antibody complexes that coengage FcεRI and FcγRIIB on basophils.<sup>15,16</sup> Passively administered IgG antibodies

or bispecific molecules capable of coligating, either directly<sup>18</sup> or indirectly,<sup>19,20</sup> FcεRI with FcγRIIB have been generated, some of which have a modified Fc portion that has a markedly increased affinity for FcγRIIB.<sup>22</sup> 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 immune checkpoint inhibitors<sup>37</sup> has been possible because regulatory mechanisms that control anticancer immunity were not impaired in patients with cancer. Therefore our study encourages development of immune checkpoint modifiers against allergic and autoimmune diseases.

### Key messages

- 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 patients.
- Intrinsic defects in mechanisms that control basophil activation do not account for the cause of allergic diseases.
- The finding that no intrinsic abnormality dampens mechanisms that control basophil activation in allergic patients encourages development of immune checkpoint modifiers against allergies.

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