

## Letter to the Editor

### Thymic stromal lymphopoietin does not activate human basophils

To the Editor:

Basophils exhibit nonredundant effector functions that contribute to the development of type 2 inflammation.<sup>1</sup> Even if IL-3 is the major basophil-activating cytokine, recent data indicated that mouse basophils can also be activated by thymic stromal lymphopoietin (TSLP).<sup>2</sup> TSLP is a member of the IL-2 family of cytokines that is thought to play important roles in human allergic diseases by instructing dendritic cells (DCs) to induce T<sub>H</sub>2 responses.<sup>3</sup> TSLP binds to a heterodimeric receptor composed of the thymic stromal lymphopoietin receptor (TSLPR) and IL-7R $\alpha$  (CD127) chains, and mainly signals through STAT5 phosphorylation (P-STAT5).<sup>3</sup> TSLP has been shown to promote the expansion of phenotypically and functionally distinct basophil subsets in mice.<sup>2</sup> Indeed, TSLP-elicited basophils differ from classical IL-3-elicited basophils by preferentially responding to IL-33 rather than IgE-cross-linking.<sup>2,4</sup> In humans, TSLP has been shown to promote the differentiation of human CD34<sup>+</sup> cells into eosinophils and basophils,<sup>5</sup> and a recent report has suggested that blood basophils from allergic patients respond to TSLP.<sup>6</sup> However, whether normal basophils from healthy donors (HDs) also respond to TSLP is unknown.

To address this question, we compared the effect of IL-3 and TSLP on purified blood basophils from HDs. An induction of CD203c, CD69, and CD63 was observed when basophils were cultured with IL-3 but not when basophils were cultured with TSLP (Fig 1, A; see Fig E1, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). In addition, basophils produced IL-4, IL-6, IL-13, CCL2/monocyte chemoattractant protein-1, CCL3/macrophage inflammatory protein-1 $\alpha$ , and TNF- $\alpha$  on IL-3 stimulation, whereas no significant secretion of these mediators nor of IL-3 was induced by TSLP (Fig 1, B). As a positive control of TSLP-mediated activation, we used purified blood myeloid DCs (mDCs) from the same donor and confirmed that TSLP induced their expression of CD83 and CD86 (Fig 1, C, and Fig E1, B) as well as secretion of IL-3, TNF- $\alpha$ , macrophage inflammatory protein-1 $\alpha$ , and monocyte chemoattractant protein-1 (Fig E1, C). We then thought to assess whether TSLP could readily signal through TSLPR/IL7R $\alpha$  in basophils and analyzed P-STAT5 expression by flow cytometry (FCM) (see Fig E2, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). Although IL-3 induced an early and persistent P-STAT5 expression in basophils, no P-STAT5 was detected on TSLP stimulation (Fig 1, D). In contrast, both IL-3 and TSLP induced P-STAT5 in mDCs (Fig 1, D). We then assessed whether basophils expressed a functional TSLP receptor. As far as we know, this question has never been addressed. We analyzed the expression of TSLPR and IL-7R $\alpha$  by FCM on basophils and mDCs from HDs (Fig 1, E). Unstimulated basophils and mDCs did not express IL-7R $\alpha$  or TSLPR. Culture of mDCs induced the expression of both TSLPR and IL-7R $\alpha$  on 40% of cells, which was further increased to 65% by IL-3 (Fig 1, E). Although TSLPR was induced in basophils on culture with IL-3, we could not detect any IL-7R $\alpha$  expression (Fig 1, E).

Similar results were obtained after intracellular staining (data not shown). Because TSLPR in basophils was induced by IL-3, we assessed whether a 24-hour priming by IL-3 could render them responsive to TSLP. Even in these conditions, however, no significant induction of P-STAT5 (Fig E2, B) or surface activation markers (data not shown) could be detected in response to TSLP.

We then sought to determine whether basophils could respond to TSLP in the context of allergy as shown recently by others.<sup>6</sup> For this purpose, we developed a whole blood assay that allowed simultaneous assessment of mDC and basophil responses in the same sample. Blood samples obtained from 8 patients with demonstrated allergy to a pneumallergen (see Table E1 in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)) and HDs were stimulated with IL-3, TSLP, or an anti-Fc $\epsilon$ RI antibody for 6 hours and analyzed by FCM. Basophils and mDCs were identified in lineage-negative CD45<sup>+</sup> cells as HLA-DR<sup>-</sup>CCR3<sup>high</sup>CD11c<sup>low</sup> and HLA-DR<sup>high</sup>CCR3<sup>-</sup>CD11c<sup>high</sup> cells, respectively (Fig 2, A). IL-3 but not TSLP induced P-STAT5 (Fig 2, B; see Fig E3, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)), CD203c expression, and CCR3 downregulation (Fig 2, C, and Fig E3, B) on blood basophils from both HDs and allergic patients. We also observed a tendency to increased responsiveness of basophils from allergic patients to Fc $\epsilon$ RI cross-linking (Fig 2, C). In contrast, both IL-3 and TSLP induced P-STAT5 in mDCs with no differences between HDs and allergic patients (Fig 2, B, and Fig E3, A). We finally assessed the expression of TSLPR and IL-7R $\alpha$  chains on basophils and mDCs using the same assay. We observed an upregulation of TSLPR and IL-7R $\alpha$  on DCs on culture of blood with medium, IL-3, or TSLP with no difference between HDs and allergic patients (Fig 2, D, and Fig E3, C). IL-3 and Fc $\epsilon$ RI cross-linking but not TSLP induced the expression of TSLPR on a small fraction of basophils with no difference between HDs and allergic patients (Fig 2, D, and Fig E3, D). Nevertheless, we did not observe any IL-7R $\alpha$  expression on basophils from either HDs or allergic patients in any of these conditions (Fig 2, D). We further confirmed these data by RT-PCR on purified basophils from allergic patients (see Fig E4, A, in this article's Online Repository at [www.jacionline.org](http://www.jacionline.org)). We concluded that blood basophils from both HDs and our allergic patients do not express IL-7R $\alpha$  and do not respond to TSLP.

Our results contrast with those published recently by another group that showed a responsiveness of isolated blood basophils from allergic patients to TSLP.<sup>6</sup> Of note, we also isolated blood basophils from allergic patients according to this study, and confirmed their lack of response to TSLP and of IL-7R $\alpha$  expression (Fig E4, B and C). In the report from Salter et al,<sup>6</sup> TSLP was shown to activate purified basophils after 1-hour stimulation, contrasting with the rather late kinetics of TSLPR expression we observed on basophils. In addition, Salter et al<sup>6</sup> reported increased CCR3 expression on basophils following IL-3, TSLP, or anti-IgE stimulation, whereas previous literature indicates that basophil activation is associated with a downregulation of CCR3 expression,<sup>7</sup> a finding we confirmed here. Whether these discrepancies are related to differences in patients included in both studies remains to be explored.

In conclusion, our results suggest that, in contrast to murine basophils, human basophils do not respond to TSLP, likely

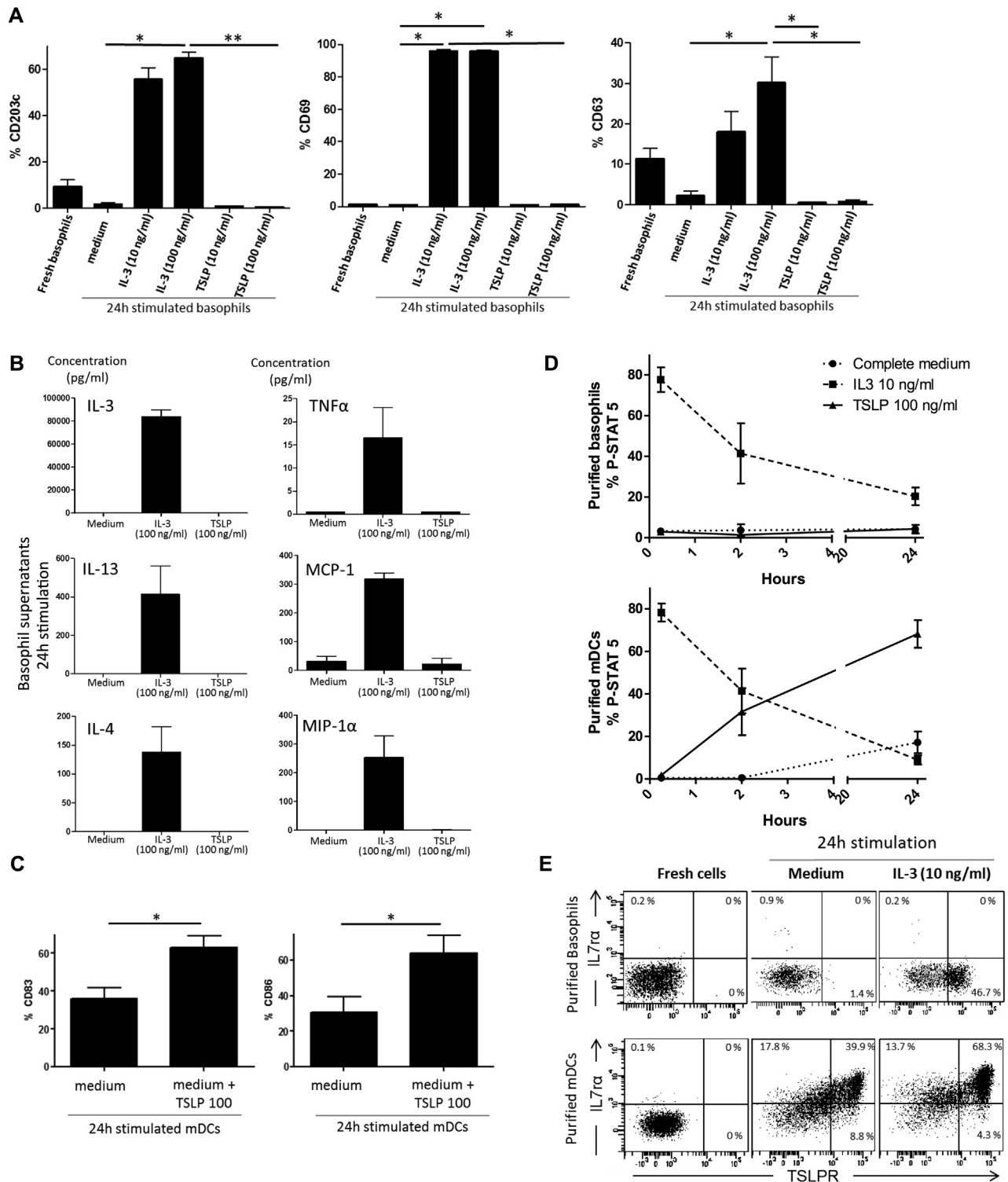


**FIG 1.** TSLP does not activate purified blood basophils from HDs. Blood basophils and mDCs were isolated from HDs and cultured with medium, IL-3, or TSLP for expression of CD63, CD69, and CD203c on basophils ( $n = 4-7$ ) (A). Cytokine and chemokine concentration in basophil supernatants ( $n = 4$ ) (B). Expression of CD83 and CD86 on mDCs ( $n = 6$ ) (C). Kinetics of P-STAT5 expression in basophils (top) and mDCs (bottom) ( $n = 3$ ) (D). TSLPR and IL-7R $\alpha$  expression on basophils (top) and mDCs (bottom) (E). MCP-1, Monocyte chemoattractant protein; MIP-1 $\alpha$ , macrophage inflammatory protein-1 $\alpha$ . Data are shown as mean  $\pm$  SEM. \* $P < .05$  and \*\* $P < .01$ .

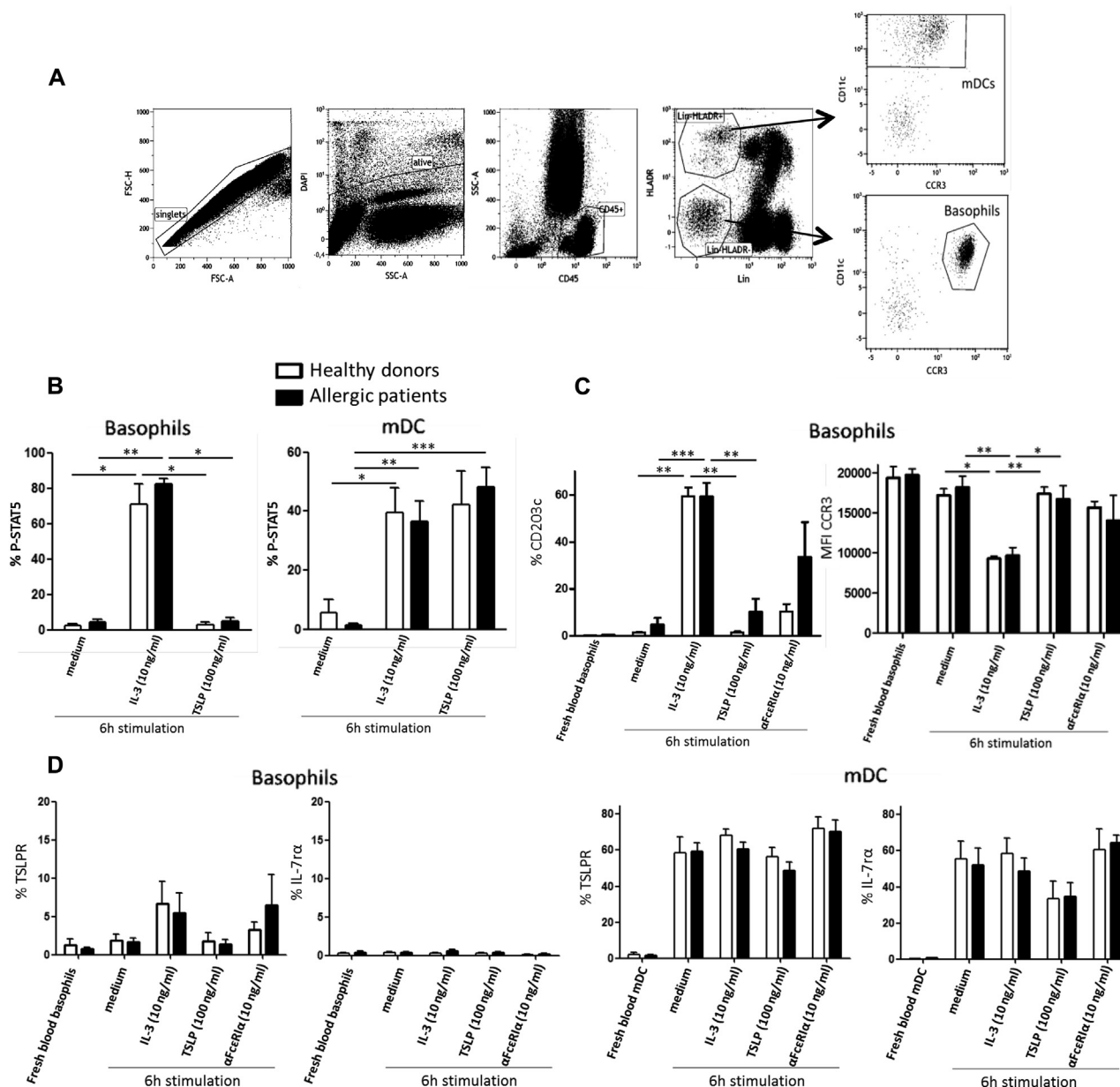


**FIG 2.** Basophils from allergic patients do not respond to TSLP and do not express IL-7Rα. Whole blood samples from HDs (empty bars, n = 5) and allergic patients (black bars, n = 8) were stimulated for 6 hours with IL-3, TSLP, anti-FcεRI antibody, or medium and analyzed by FCM. Gating strategy on whole blood (A). Intracellular P-STAT5 expression in basophils (left) and mDCs (right) (B). CD203c (left) and CCR3 (right) expression on basophils (C). % of IL-7Rα- and TSLPR- expressing basophils (left) or mDCs (right) (D). Data are shown as mean ± SEM. \*P < .05 and \*\*P < .01.

because of the absence of IL-7Rα expression. Expression of TSLPR by human basophils in the absence of IL-7Rα is in fact intriguing and whether TSLPR could associate in these cells with a chain distinct from IL-7Rα deserves further investigation.

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## METHODS

### Basophils and mDCs isolation

PBMCs were isolated by Ficoll density gradient centrifugation (Ficoll-Paque PLUS, GE Healthcare, Velizy, France) of leukapheresis products obtained from HDs on written informed consent and approval by the Institutional Review Board (Etablissement Français du Sang Pays de la Loire, Nantes, France). Basophils and mDCs were isolated by immunomagnetic depletion using, respectively, Basophil isolation kit II human and mDC isolation kit (Miltenyi Biotec, Paris, France) with the autoMACS pro-separator (Miltenyi Biotec). Alternatively, basophils were isolated with a kit from STEMCELL technologies (Grenoble, France) as in Salter et al.<sup>E1</sup>

### Culture of basophils and mDCs

Basophils and mDCs were cultured in complete medium (RPMI [Sigma-Aldrich, Lyon, France] supplemented with 10% FBS, 1% essential amino acid, 1% HEPES, 1% sodium pyruvate, 1% penicillin-streptomycin, 1% glutamine) at  $10^6$  cells/mL in 96-well plates with or without IL-3, TSLP (R&D system, Minneapolis, Minn), anti-IgE antibody (Beckman Coulter, Villepinte, France), or anti-FcεRI antibody (Miltenyi Biotec) at different concentrations.

### Flow cytometry

Purity of basophils was assessed by staining cells with Lin-fluorescein isothiocyanate (FITC), HLADR-allophycocyanin-cyanin 7 (APCCy7), CD123-phycoerythrin-cyanin 5.5 (PeCy5.5) (BD Biosciences, Le Pont de Claix, France), and CCR3-APC (eBiosciences, San Diego, Calif), and the purity of mDCs was assessed by staining cells with Lin-FITC, HLADR-APCCy7, CD123-PeCy5.5 (BD Biosciences), and CD11c-PeCy7 (Beckman Coulter) analyzed on a BD FACSCanto II Cytometer (BD Biosciences).

To evaluate cell activation, basophils were stained with CD69-PeCy7, CD63-FITC (BD Biosciences), and CD203c-APC (Miltenyi Biotec) and mDCs with CD83-PE and CD86-APC (BD Biosciences). Staining with TSLPR-APC or IgG<sub>1</sub>-APC (Biolegend, San Diego, Calif) and CD127-PE or IgG<sub>1</sub>-PE (Beckman Coulter) was also performed.

The expression of P-STAT5 was analyzed by flow cytometry using P-STAT5 APC (BD Biosciences) after cell fixation and permeabilization with Fix buffer I and Perm Buffer III (BD Biosciences).

### Cytokines and chemokines

Supernatants of basophils and mDC cultures were collected after 24-hour stimulation with IL-3, TSLP, or medium alone (as negative control) and stored at  $-80^{\circ}\text{C}$  until use. IL-3, IL-4, IL-13, monocyte chemoattractant protein-1, macrophage inflammatory protein-1α, and TNF-α concentrations were measured using a multiplex fluorescent bead-based immunoassay (Thermo Fisher Scientific, Villebon-sur Yvette, France) with Luminex's xMAP instruments (Magpix, Luminex, Hertogenbosch, The Netherlands).

### Whole blood assay

Peripheral blood was sampled in EDTA tubes from HDs and allergic patients with a positive skin test result or specific IgE to aeroallergen, selected in the Department of Allergology of Nantes University Hospital (Table E1). All subjects provided signed informed consent. Three hundred microliter of whole blood was stimulated with IL-3, TSLP, or anti-FcεRI antibody (Miltenyi Biotec) for 6 hours. Cells were then stained with CD45-Kro, CD11c-PECy7 (Beckman Coulter), 4'-6-diamidino-2-phenylindole (ThermoFisher Scientific, Waltham, Mass), Lin-FITC, HLADR-APCCy7, CCR3-PerCPCy5.5, CD86 PE (BD Biosciences), and CD203c-APC (Miltenyi Biotec) or TSLPR-APC (Biolegend) and CD127-PE (Beckman Coulter). P-STAT5 was also assessed using P-STAT5 APC (BD Biosciences) and the prefix expose kit (Beckman Coulter).

### Real-time quantitative PCR

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) according to manufacturer's instructions. Reverse transcription was performed using Murine Moloney Leukemia Virus Reverse Transcriptase (Thermo Fisher Scientific) following manufacturer's instructions. Gene expression was assessed with the TaqMan Fast Advanced Master Mix reagent (Applied Biosystems, Foster City, Calif). Primers and probes were purchased from Applied Biosystems (see Table for complete list). Real-time PCR was performed using the StepOne Plus System (Applied Biosystems). Then, relative expression was normalized to hypoxanthine-guanine phosphoribosyltransferase and calculated using  $2^{-\Delta\Delta C_t}$ . Results were expressed in arbitrary units.

| Target | Gene         | Reference     | Furnisher          |
|--------|--------------|---------------|--------------------|
| TSLPR  | <i>CRLF2</i> | Hs00845692_m1 | Applied Biosystems |
| IL7Rα  | <i>IL7R</i>  | Hs00902334_m1 | Applied Biosystems |
| HPRT   | <i>HPRT1</i> | Hs99999909_m1 | Applied Biosystems |

### Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, San Diego, Calif). Multiple group comparisons were carried out using nonparametric Kruskal-Wallis test (differences were defined as statistically significant when  $P < .05$ ) and then Dunn's multiple comparison posttest ( $*P < .05$ ,  $**P < .01$ ,  $***P < .001$ ).

### REFERENCE

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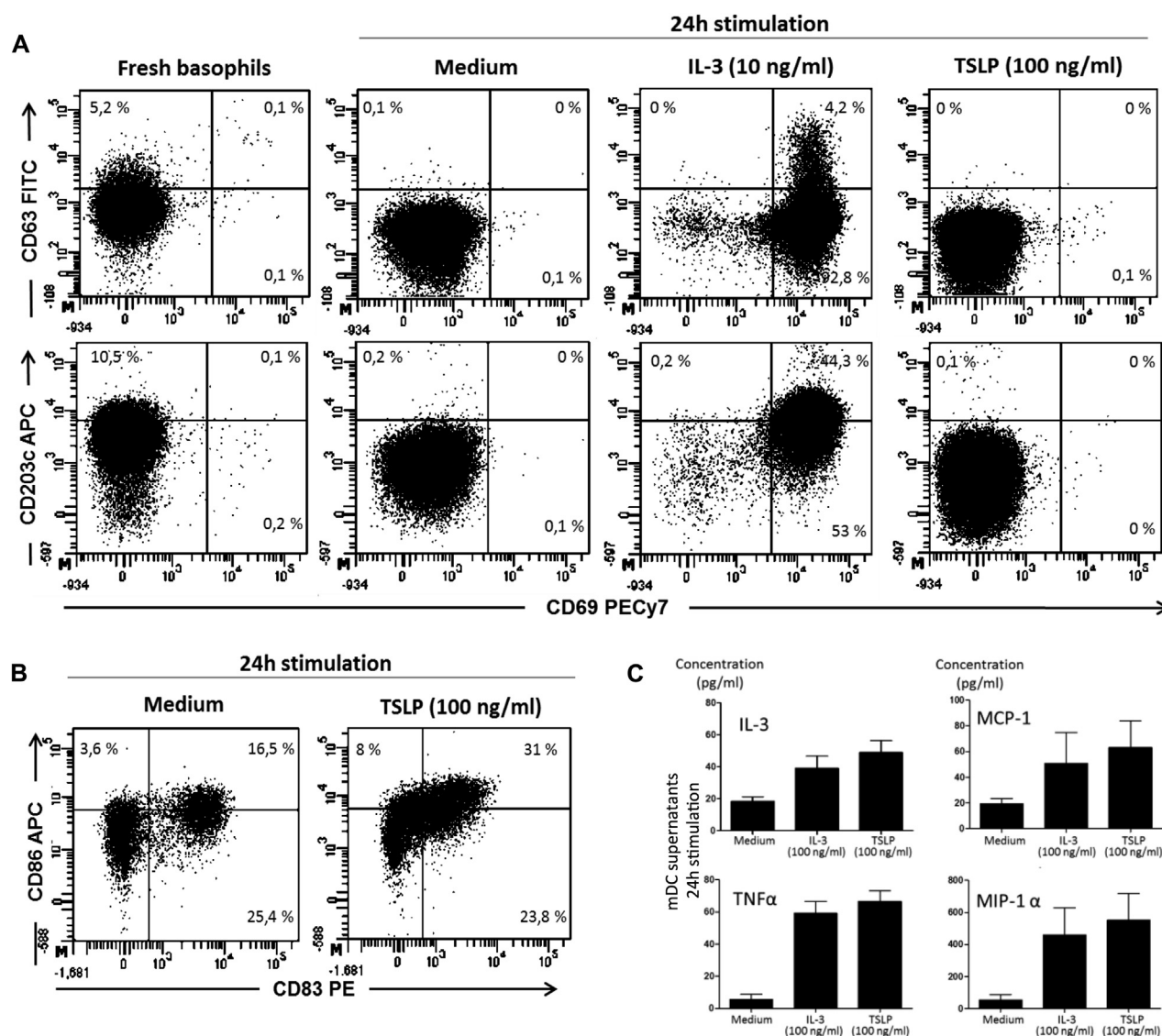


**FIG E1.** **A**, CD63, CD69, and CD203c expression on freshly isolated basophils and after 24-hour stimulation with medium, IL-3, or TSLP. **B**, CD86 and CD83 expression on isolated mDCs after 24-hour stimulation with medium or TSLP. **C**, Cytokine secretion by isolated mDCs after 24-hour stimulation by medium, IL-3, or TSLP and measured by a multiplex assay. *MCP-1*, Monocyte chemoattractant protein; *MIP-1 $\alpha$* , macrophage inflammatory protein-1 $\alpha$ .

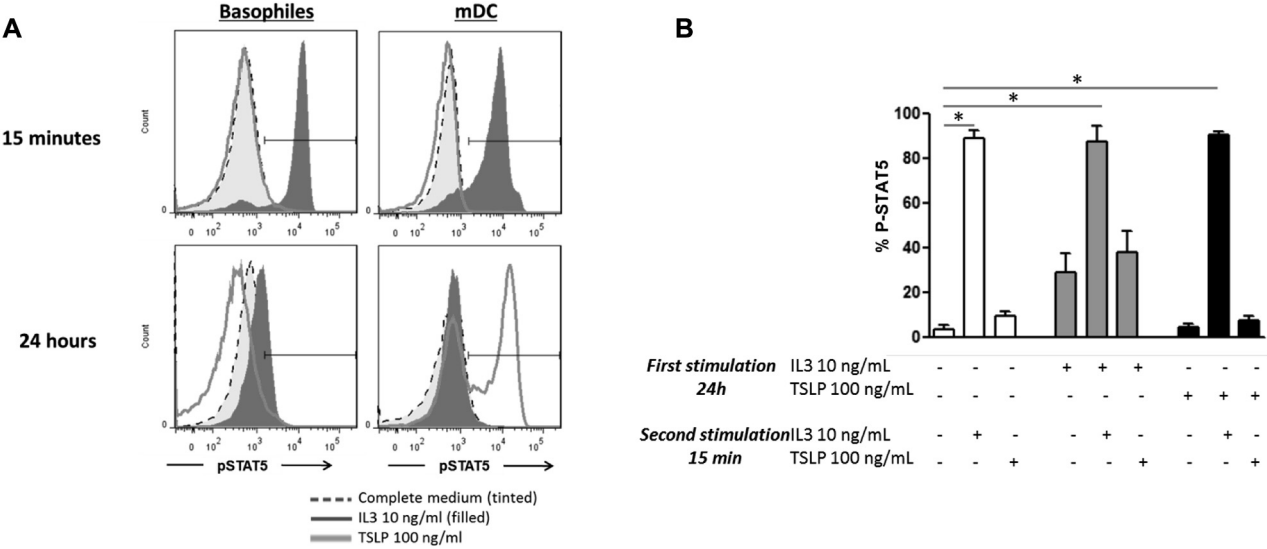


**FIG E2. A,** Histograms of P-STAT5 expression in purified basophils and mDCs after stimulation by IL-3 or TSLP. **B,** P-STAT5 expression in basophils after 24-hour priming followed by 15-minute stimulation with IL-3 or TSLP (n = 3-5 donors).

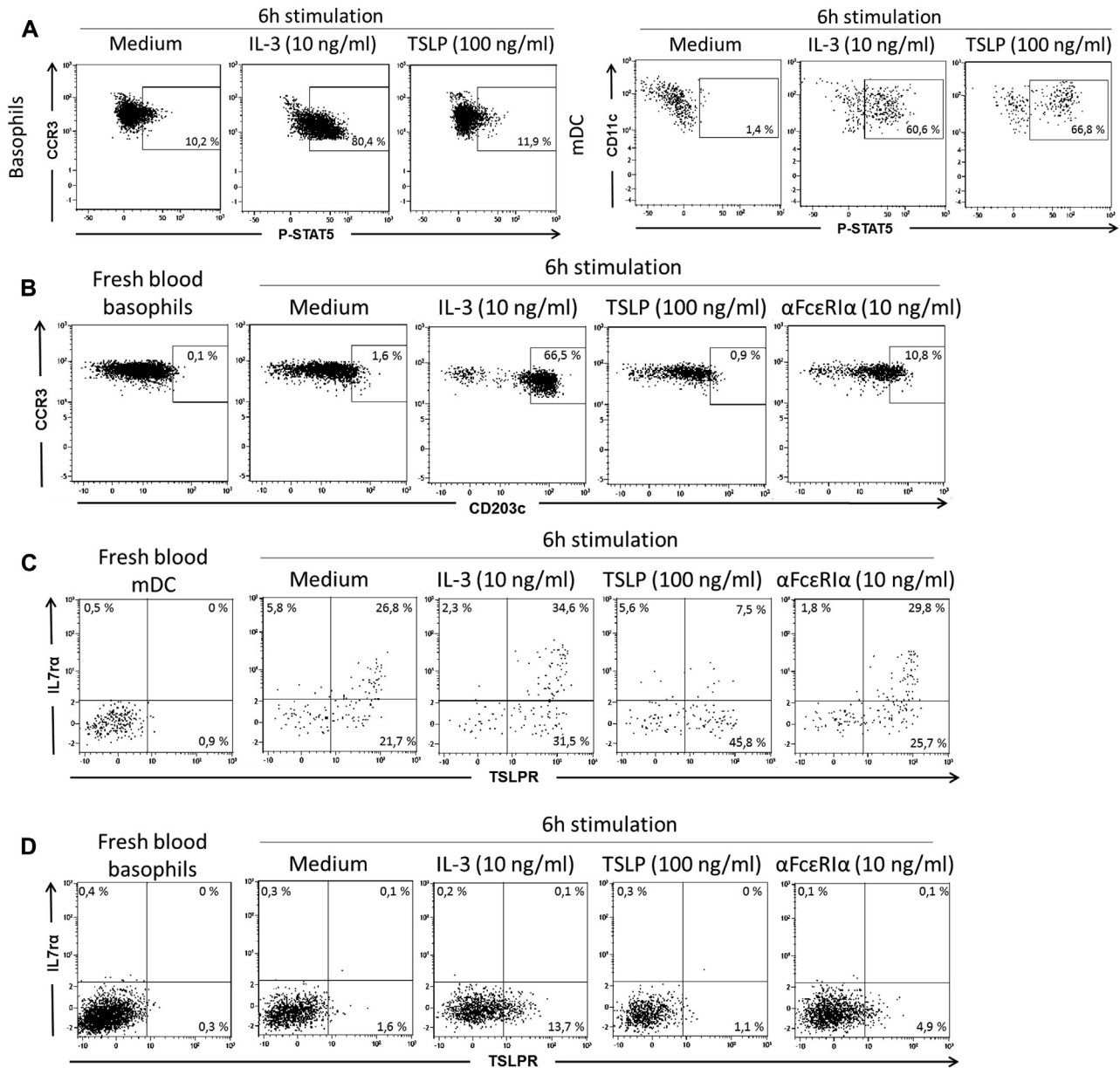


**FIG E3.** Intracellular P-STAT5 expression in basophils and mDCs (**A**) in whole blood samples from an allergic donor, after 6-hour stimulation with IL-3, TSLP, or medium. CD203c and CCR3 expression on basophils (**B**) and IL-7 $\alpha$  and TSLPR expression on mDCs (**C**) or basophils (**D**) in whole blood samples from an allergic donor, either fresh or after 6-hour stimulation with IL-3, TSLP, anti-Fc $\epsilon$ RI antibody, or medium.

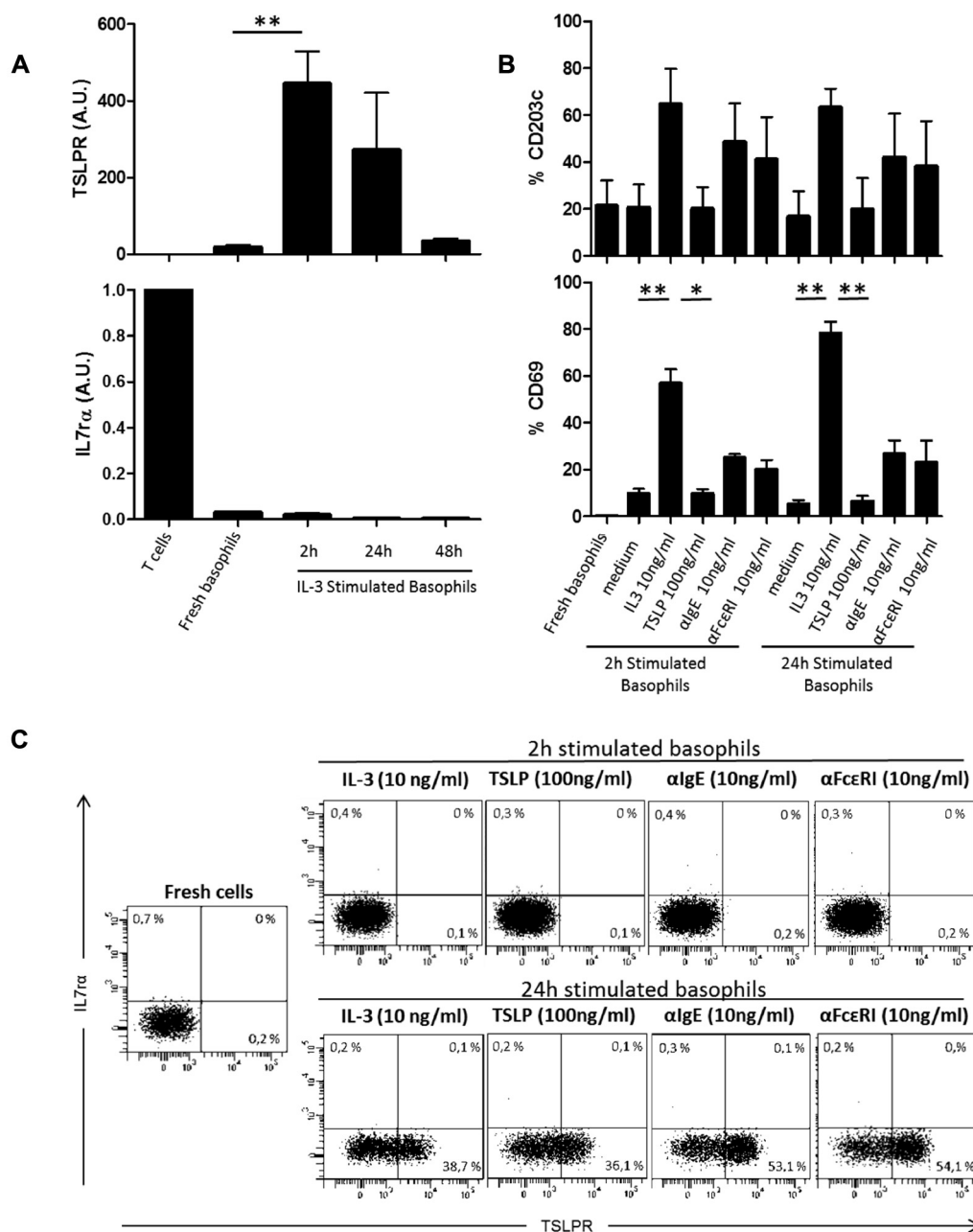


**FIG E4. A**, TSLPR and IL7R mRNA expression assessed by RT-PCR in fresh and IL-3-activated basophils from allergic patients ( $n = 3-5$  per time point). **B**, CD203c and CD69 expression on blood basophils isolated from allergic patients using the Stem Cell Technologies kit, either fresh or after culture with medium, IL-3, TSLP, anti-IgE mAb, or anti-FcεRI mAb for 2 hours or 22 hours ( $n = 3-5$  per time point/condition). **C**, TSLPR and IL-7Rα expression on blood basophils isolated from allergic patients using the Stem Cell Technologies kit, either fresh or after culture with IL-3, TSLP, anti-IgE mAb, or anti-FcεRI mAb for 2 hours or 22 hours. Similar results were obtained in 5 different allergic individuals. A.U., Arbitrary unit.

**TABLE E1.** Allergic patients' details

| Patient no. | Sex | Age (y) | Clinical manifestation main (secondary) | Severity | Treatment (at the time of sampling) |
|-------------|-----|---------|-----------------------------------------|----------|-------------------------------------|
| 1           | F   | 35      | AR, AD                                  | Mild     | None                                |
| 2           | M   | 30      | AD (AR)                                 | Moderate | Antihistamine                       |
| 3           | M   | 54      | AA (AD)                                 | Moderate | Antihistamine, steroid              |
| 4           | M   | 22      | AD (AA)                                 | Severe   | Antihistamine, steroid              |
| 5           | M   | 35      | AR                                      | Moderate | Antihistamine, steroid              |
| 6           | F   | 23      | AR                                      | Moderate | None                                |
| 7           | F   | 24      | AR                                      | Mild     | None                                |
| 8           | F   | 56      | AR                                      | Mild     | Antihistamine                       |
| 9           | F   | 29      | AR                                      | Mild     | None                                |
| 10          | F   | 24      | AR                                      | Mild     | None                                |
| 11          | M   | 33      | AR                                      | Mild     | None                                |
| 12          | F   | 35      | AR, AA                                  | Mild     | Antihistamine                       |
| 13          | F   | 36      | AR, AA                                  | Mild     | None                                |
| 14          | F   | 38      | AR, AA                                  | Mild     | Antihistamine                       |
| 15          | M   | 32      | AR                                      | Mild     | None                                |
| 16          | F   | 28      | AR, AA (AD)                             | Mild     | None                                |

AA, Allergic asthma; AD, atopic dermatitis; AR, allergic rhinitis; F, female; M, male; ND, not determined.